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<b>(54) Title:</b> BOVINE FOOTROT TREATMENT AND PREVENTION  <b>(57) Abstract</b>  This invention provides compositions and methods for treating or preventing footrot, in particular bovine footrot, by administering <i>Porphyromonas</i> and/or <i>Prevotella</i> and/or subunits and/or toxins thereof or neutralizing agents such as antibodies thereto. A model useful for evaluating the effectiveness of footrot treatments or preventatives is also provided.			
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## BOVINE FOOTROT TREATMENT AND PREVENTION

5

### FIELD OF THE INVENTION

The present invention relates to compositions useful for the treatment and prevention of footrot and, in particular, bovine footrot.

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### BACKGROUND OF THE INVENTION

Acute bovine footrot, also known as interdigital phlegmon or acute interdigital phlegmon (AIP), is a common infection in cattle [1,11]. The actual prevalence of this disease in many types of cattle is not fully described; however, in some years the prevalence in feedlot animals can reach 10-25% if preventative measures such as feeding antibiotics are not widely implemented.

It is an anaerobic bacterial infection characterized by acute inflammation which is manifested as tissue edema and local infiltration of subcutaneous tissues with polymorphonuclear granulocytic neutrophils (PMN) [16]. Typically this disease involves necrosis of the interdigital epidermis and the underlying dermis. Very often there is an ascending cellulitis which can result in severe swelling from the coronet to the fetlock joint. This infection, if left untreated, can result in sequelae such as septic joint involvement which can lead to euthanasia [2].

The infection is thought to be caused by a synergistic association of anaerobic bacteria including *Bacteroides melaninogenicus*, *Fusobacterium necrophorum* [12], and possibly other bacteria such as *Dichelobacter (Bacteroides) nodosus* [13] or *Actinomyces pyogenes* [14]. Recently *B.melaninogenicus* has been divided into several distinct species of bacteria including *Porphyromonas* sp and *Prevotella* sp [4]. *Fusobacterium necrophorum* and *B.melaninogenicus* have been previously used

together to experimentally infect cattle [12]. That investigation did not examine microscopic pathology or the minimum inhibitory concentrations [MIC's] and minimum bactericidal concentrations [MBC's] of the antibiotic(s) for the pathogens used in the experimental infection.

5           Several treatments have been advocated for acute bovine footrot including penicillin [11], oxytetracycline [15], cephalosporins [16], and sulfonamides [11].

          Parenteral antibiotic treatment of individual clinically affected animals is effective for treating cattle that can be easily handled and frequently observed. Therapy in fat cattle (i.e., animals nearly ready for marketing as beef) is complicated by the fact  
10   that many of these drugs cannot be used without delaying slaughter. Antibiotics without withdrawal periods are available for these animals; however, the expense remains significant. Currently, recommended therapy for these animals can involve daily treatment for up to five days or longer. Cephalosporin, a  $\beta$ -lactam antibiotic, is one recommended therapeutic regimen. For a 500 kg steer, cephalosporin therapy for  
15   five days can approach \$50 in antibiotic cost alone for a single episode of the disease. This clearly does not include the costs of manpower for giving the treatments, the costs of lost production (e.g., reduced weight gain) in affected cattle, or the significant animal suffering that occurs as a result of this infectious lameness.

          There are potentially devastating effects of this disease in breeding bulls if the  
20   infection occurs during breeding season and libido is reduced. Conventional antibiotic therapy in mature breeding bulls is problematic because of the frequency of treatments and the massive volume of antibiotic required, in addition to the cost of antibiotics. Although infection can be minimized in some types of cattle through feeding antibiotics, fat cattle are again at high risk because of our inability to use these drugs  
25   due to withdrawal times. Preventative measures, such as footbaths, are recommended in many parts of the world but are not practical under many circumstances. The potential environmental implications of using compounds such as blue stone and formaldehyde are also a consideration.

          Animals affected with AIP are believed to develop immunity, but the importance  
30   of this immunity is unclear [2]. In many acute infectious inflammatory diseases

phagocytosis by polymorphonuclear granulocytic neutrophils (PMN) is a central mechanism in the resolution of infection, but these cells have never been specifically evaluated in the context of acute bovine footrot. Specific immunity to etiologic agents of footrot also may be important in resolution of the infection [2]; however, studies  
5 have yet to be conducted on precisely how these cells are involved in the mechanisms of this process.

The development of a therapeutic agent and/or a vaccine, additional tools for cattlemen to minimize the effects of footrot, would be worthwhile and a significant contribution to sustainable agricultural practices.

10 Traditionally, *Fusobacterium necrophorum* has been described as the cause of bovine footrot [2-4]. Vaccines based on this microorganism are known and/or available [5,8,9], but efficacy is questionable and use is not broad. Use of 6-substituted 3-nitroimidazo[1,2,b]pyridazine for the control of footrot and liver lesions caused by *Fusobacterium necrophorum* has also been disclosed [10].

15

### SUMMARY OF THE INVENTION

This invention provides a method of preventing or treating bovine footrot by administering vaccine compositions comprising at least one of the causative agents of bovine footrot, i.e., *Porphyromonas* and *Prevotella* and/or subunits and/or toxins  
20 thereof. Compositions comprising *Porphyromonas levii* and *Prevotella intermedia* are preferred. Protectively immunogenic vaccine compositions may also contain a specific bovine immunoglobulin G<sub>2</sub> (IgG<sub>2</sub>) destroying toxin of *Porphyromonas levii* and a  $\beta$ -lactamase enzyme of *Prevotella intermedia*, either or both of which can be inactivated and included in the vaccine composition. The invention also provides a method of  
25 treating or preventing bovine footrot by administering compositions comprising at least one neutralizing agent, e.g., an antibody, to a causative agent of bovine footrot, i.e., *Porphyromonas* and *Prevotella* and/or subunits and/or toxins thereof. Compositions comprising antibodies to *Porphyromonas levii* and *Prevotella intermedia* and/or their toxin(s) or subunit(s) are preferred. Effective compositions may contain antibodies to  
30 immunoglobulin proteases, including a specific bovine immunoglobulin G<sub>2</sub> (IgG<sub>2</sub>)

destroying toxin of *Porphyromonas levii* and/or antibodies to antibiotic resistance enzymes, including a  $\beta$ -lactamase enzyme of *Prevotella intermedia*.

Our isolation techniques and our immunology studies suggest that *Porphyromonas* (and especially *P. levii*) and *Prevotella* (and especially *P. intermedia*)  
5 are more appropriate vaccine candidates than *Fusobacterium necrophorum*. Using surgical biopsy techniques and stringent anaerobic culture methodology, we have not isolated *Fusobacterium necrophorum* from internal infected tissues in a single case of bovine footrot.

The invention provides compositions and methods for preventing or treating  
10 infectious lameness, particularly in cattle, using the bacteria *Porphyromonas* and/or *Prevotella*, toxins isolated from these bacteria, and neutralizing agents which may be used in the compositions. The invention provides methods of preparing and methods of using a bacterial protease toxin, an antibiotic destroying toxin, and antibodies to the toxins.

15 Accordingly, in one aspect, the invention provides compositions for preventing or treating footrot comprising *Porphyromonas* and/or *Prevotella*. Compositions comprising subunit(s) and/or toxin(s) of these bacteria are also provided, as are methods of preparing these compositions.

A further aspect of the invention is a method of preventing or treating infection  
20 in an animal comprising administering to the animal an effective amount of a composition comprising *Porphyromonas* and/or *Prevotella* and/or subunit(s) and/or toxin(s) thereof.

Another aspect of the invention provides isolated toxins from *Porphyromonas* and/or *Prevotella*. Methods of preparing the toxins are also provided, as are antibodies  
25 to the toxins and a method of passive immunization using the antibodies.

Yet another aspect of the invention provides an experimental model useful for evaluating the effectiveness of possible preventatives or cures for bovine footrot comprising a bovine which has been administered *Porphyromonas* or *Prevotella*, especially in conjunction with *Fusobacterium necrophorum*.

In yet a further aspect, the invention provides compositions for preventing or treating footrot comprising at least one agent which neutralizes *Porphyromonas* and/or *Prevotella* and/or subunit(s) and/or toxin(s) thereof.

In a still further aspect, the invention provides methods for treating or  
5 preventing footrot comprising administering to an animal suffering from or susceptible to footrot an effective amount of a composition which enhances neutrophil-mediated resolution of infection by bacteria associated with footrot.

### BRIEF DESCRIPTION OF THE DRAWINGS

10 Figs. 1A through 1D are electrophoretic transfers of biotin labeled immunoglobulins that have been incubated with IgG<sub>2</sub> destroying toxin-containing extract from *Porphyromonas levii*. In Fig. 1A IgG was used as the immunoglobulin; in Fig. 1B IgM was used; in Fig. 1C IgG<sub>1</sub> was used; and in Fig. 1D IgG<sub>2</sub> was used. Note that fragments of immunoglobulins are present in Figs. 1A and 1D, but not in Figs. 1B  
15 and 1C, showing that this protease is IgG<sub>2</sub> specific.

Fig. 2 is a photograph illustrating the cefinase method of detecting  $\beta$ -lactamase enzyme. The upper disc is a disc exposed to *Prevotella intermedia*, the lower left disc a negative control, and the lower right disc (marked  $\beta$ ) is exposed to commercially purified  $\beta$ -lactamase enzyme-positive control. The results show that *Prevotella*  
20 *intermedia* produces a  $\beta$ -lactamase enzyme.

Fig. 3 is a silver stain SDS-polyacrylamide gel of varying concentrations of protein from a 40-50% ammonium sulfate precipitation of extracts from *Prevotella intermedia*. These extracts retained potent  $\beta$ -lactamase activity as assessed by the cefinase and other assays.

25 Figs. 4A through 4C are Western immunoblots of the reaction of bacterial antigens of *Fusobacterium necrophorum* (Fig. 4A), *Prevotella intermedia* (Fig. 4B), and *Porphyromonas levii* (Fig. 4C) with serum collected from cattle experimentally infected with footrot. The results show that animals developed immunity, as evidenced by the development of serum antibodies to *Prevotella intermedia* and *Porphyromonas*  
30 *levii*, but not to *Fusobacterium necrophorum*, after experimental infection.

Figs. 5A through 5D are Western immunoblots of the reaction of bacterial antigens from *Fusobacterium necrophorum* (FN), *Prevotella intermedia* (5.2), and *Porphyromonas levii* (7.5) with acute serum - taken the day clinical signs were noticed (A); and convalescent serum - taken 28 days after appearance of clinical signs (C) from  
 5 four animals (Figs. 5A through 5D) naturally infected with bovine footrot. Note the generally minimal response to *Fusobacterium necrophorum* and the increasing intensity of banding patterns to either *Prevotella intermedia* or *Porphyromonas levii*.

Fig. 6A is a graphical representation of the footrot scores in Group 1 animals (infected with *Porphyromonas levii* and *Fusobacterium necrophorum*). Data are mean  
 10 scores  $\pm$  standard errors. The arrow indicates the time of treatment and the asterisks represent a statistically significant ( $P < 0.05$ ) reduction in footrot score compared to pre-treatment score.

Fig. 6B is a graphical representation of the footrot scores in Group 2 animals (infected with *Prevotella intermedia* and *Fusobacterium necrophorum*). Data are mean  
 15 scores  $\pm$  standard errors. The arrow indicates the time of treatment and the asterisks represent a statistically significant ( $P < 0.05$ ) reduction in footrot score compared to pre-treatment score.

Fig. 7 is a graphical representation of the footrot scores of animals (combined data of Group 1 and Group 2). Data are mean scores  $\pm$  standard errors. The arrow  
 20 indicates the time of treatment and the asterisks represent a statistically significant ( $P < 0.05$ ) reduction in footrot score compared to pre-treatment score.

Fig. 8 is a graphical representation of the footrot scores of animals (combined data of Group 1 and Group 2 - excluding values from a non-responding animal). Data are mean scores  $\pm$  standard errors. The arrow indicates the time of treatment and the  
 25 asterisk represents statistical significance compared to pre-treatment score ( $P < 0.05$ ).

Fig. 9A is a nitrocellulose transfer of biotinylated IgG exposed to culture supernatant of *P. levii*. From left to right are increasing times (hours) of exposure. Lane A = 0, Lane B = 0.5, Lane C = 1, Lane D = 2, Lane E = 4, Lane F = 8, Lane G = 24, Lane H = 48, and Lane I = 72h time samples, showing increasing



intensity of the degradation bands smaller than the heavy and light chains as incubation time increases and the near complete degradation of the heavy chain by 24h.

Fig. 9B is a nitrocellulose transfer of biotinylated IgM exposed to culture supernatant of *P. levii*. From left to right are increasing times (hours) of exposure.

- 5 Lane A = 0, Lane B = 0.5, Lane C = 1, Lane D = 4, Lane E = 8, Lane F = 24, Lane G = 48, and Lane H = 72h time samples, showing the absence of degradation bands, with only the light and heavy chains of IgM evident.

Fig. 10A is a nitrocellulose transfer of biotinylated IgG<sub>1</sub> exposed to culture supernatant of *P. levii*. From left to right are increasing times (hours) of exposure.

- 10 Lane A = 0, Lane B = 0.5, Lane C = 1, Lane D = 2, Lane E = 4, Lane F = 8, Lane G = 24, Lane H = 48, and Lane I = 72h time samples, showing the absence of degradation bands, with only the light and heavy chains of IgG<sub>1</sub> being evident.

Fig. 10B is a nitrocellulose transfer of biotinylated IgG<sub>2</sub> exposed to culture supernatant of *P. levii*. From left to right are increasing times (hours) of exposure.

- 15 Lane A = 0, Lane B = 0.5, Lane C = 1, Lane D = 2, Lane E = 4, Lane F = 8, Lane G = 24, Lane H = 48, and Lane I = 72h time samples, showing the presence of additional bands by 0.5 h, as well as the heavy and light chains of IgG<sub>2</sub>.

#### DETAILED DESCRIPTION OF THE INVENTION

- 20 The invention is drawn in part to the unexpected discovery that the bacteria *Porphyromonas* and/or *Prevotella*, and especially *Porphyromonas levii* and *Prevotella intermedia*, and their subunits and toxins, may be used to prepare vaccine compositions and antibodies useful to prevent and treat bovine footrot. These organisms may also be used as the basis for an experimental model useful for evaluating treatments or
- 25 preventatives for the disease. The invention is also drawn in part to the discovery that administration of a composition which enhances PMN phagocytosis of the bacteria associated with footrot is a useful method to treat or prevent footrot. Agents which neutralize immunoglobulin proteases and/or the causative organisms of footrot are especially useful.

30

A. Definitions

As used herein, the following terms have the following meanings:

Adjuvant: a vehicle used to enhance antigenicity. The use of adjuvants is well-known in the art. Adjuvant may include suspensions of minerals on which antigen may  
5 be absorbed, such as alum, aluminum hydroxide or phosphate; water-in-oil emulsions in which antigen solution is emulsified in mineral oil, such as Freund's incomplete adjuvant; and may include additional factors, such as killed mycobacteria in Freund's complete adjuvant, to further enhance antigenicity.

Antibody: a molecule, especially a protein, that binds immunologically to a  
10 known antigen or a determinant of an antigen. Antibodies are also referred to as immunoglobulins, and are generally classified into isotypes (i.e., IgA, IgD, IgG, IgE and IgM) based on their physicochemical characteristics and amino acid sequence identity. IgA and IgG are further divided into subtypes (i.e., IgA<sub>1</sub>, IgA<sub>2</sub>, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub> and IgG<sub>4</sub>) based on sequence similarity.

15 Bacterin: the term used to describe vaccines containing killed or inactivated bacteria.

Effective Amount: dose required to protect an animal against infections or disease or alleviate a particular symptom of an infection or disease.

Footrot: also known as interdigital phlegmon, interdigital necrobacillosis, foot  
20 abscess, foul-in-the-foot or superfoul. A necrotizing infection of interdigital skin characterized by deep necrosis and fissures. It is associated with infection with either or both of *Porphyromonas* and *Prevotella*. The symptoms of the infection, such as lameness, swelling, edema, interdigital lesions, interdigital purulent exudate, etc. are also included in the term footrot.

25 Immune Response: development in the host of a cellular and/or antibody-mediated immune response to a composition or vaccine of interest. Such a response may consist of one or more of the following: inducing cytokine production, producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

Inactivation: no longer capable of causing disease or a disease condition.

Bacteria or bacterial toxins may be inactivated by many known methods. These include, but are not limited to: chemical treatment, e.g., formalin; heat treatment, e.g., mild heating until a toxin is inactivated or cells are killed; attenuation, e.g., making a  
5 bacterium or toxin inactive through mutation or genetic/chemical alteration; sonication, e.g., disruption of cells by exposing a suspension of the cells to high frequency sound waves; freezing, e.g., snap freezing cells until they lyse; etc.

Lactamase: also known as  $\beta$ -lactamase. An enzyme or toxin which cleaves a  $\beta$ -lactam structure such as that found in certain antibiotics. This term includes the  
10 lactamase produced by *Prevotella intermedia*.

Neutralize: able to prevent or alleviate toxic effects. An agent is a neutralizing agent if it prevents or alleviates the toxic effects of an organism, toxin or subunit. Included in this term are antibodies which bind to an organism, toxin or subunit so as to prevent or inhibit its toxic activity.

15 *Porphyromonas*: a genus of anaerobic bacterium that causes bovine footrot. As used in this application, the term includes all species of this genus. The genus *Bacteroides*, formerly used to refer to as *Porphyromonas*, is also included in this term as used in this application. In particular, *Porphyromonas levii* was formerly classified as *B. melanogenicus ssp.levii*.

20 Prevention of Symptoms: includes prevention of any effect caused by an infection or disease condition, including effects caused by a toxin.

*Prevotella*: a genus of anaerobic bacterium that causes bovine footrot. As used in this application, the term includes all species of this genus. The genus *Bacteroides*, formerly used to refer to *Prevotella*, is also included in this term as used in this  
25 application. In particular, *Prevotella intermedia* was formerly classified as *B. melanogenicus ssp. intermedia*.

Production *in vitro*: production in culture, not in an infected host animal. Production *in vitro* includes recombinant production.

Protease: an enzyme or toxin which cleaves protein. Proteases may specifically cleave certain proteins, like the IgG<sub>2</sub> specific protease produced by *Porphyromonas levii*.

Protectively Immunogenic: able to protect an animal against infection or disease  
5 or alleviate particular symptoms of an infection or disease.

Recombinantly Produced: produced by means of gene expression in any other system including microorganisms, plants or animals and/or chemically synthesized by methods known in the art when the gene sequence or amino acid sequence is known.

Resolution of Infection: arrest of infection so as to return to a normal state.  
10 Resolution of infection includes resolution of infection mediated by neutrophils, e.g., phagocytosis and oxidative metabolism.

Subunit: any part of a bacterium which is less than the whole organism.  
Subunits that are antigenic may be used in vaccine compositions to produce an immune response. Subunits may include flagella, pili, fimbriae, membranes, membrane  
15 proteins, toxins and any other part of an organism which may be antigenic and induce an immune response. This includes recombinantly produced subunits.

Toxin: a noxious or poisonous substance that is produced by a bacterium. It may be produced and released extracellularly (exotoxin). This includes recombinantly produced toxins.

20

#### B. Detailed Description of the Invention

Our studies have shown that during a typical natural clinical infection, humoral and cellular immunity are not strongly developed toward the strains of bacteria involved in natural infections of bovine footrot. Minimal response is seen by Western  
25 immunoblot to antigens from these bacteria (i.e., antibodies are poorly produced or readily destroyed). This may be because natural infection does not adequately stimulate the immune system. Alternatively, a normal immune response may be prevented by the infectious agent (e.g., the IgG<sub>2</sub> specific protease produced by *Porphyromonas levii*). Neutrophil-mediated resolution of infection includes phagocytosis of infecting bacteria  
30 and intracellular destruction of bacteria by oxidative metabolism.

Bacteria have a wide variety of mechanisms for evading or altering an immune response by a host animal. These mechanisms can enable bacteria to more readily establish and maintain an infection. Specifically, it is known that bacterial proteases, and in particular those which cleave immunoglobulins, are one such mechanism. Such proteases may modify important host defenses against bacterial infection. Organisms which produce immunoglobulin proteases include *Proteus mirabilis* [20,21], *Staphylococcus aureus* [22], *Neisseria gonorrhoeae* [23] and *Pasteurella haemolytica* [24]. It is likely that bovine IgG and IgM are the most important classes of immunoglobulins produced by cattle in response to a footrot infection. IgM is the first immunoglobulin recruited against a foreign antigen and IgG has the highest serum concentration. Both are central in immunity during tissue infections [25]. Thus, immunoglobulin protease production by *Porphyromonas levii* is likely an integral mechanism for these bacteria to evade humoral immunity during infection.

Polymorphonuclear granulocytic neutrophils are a central component of acute inflammation in animals [26], including cattle. These cells rapidly, and efficiently, phagocytose most bacteria under normal circumstances. However, we have shown that PMN do not readily phagocytose *P. levii* unless substantial amounts of specific anti-*P. levii* immunoglobulin are present. Thus, *in vivo*, specific anti-*P. levii* immunoglobulin is crucial for the resolution of infection.

Several types of immunoglobulin can be involved in enhancing phagocytosis by PMN. IgG is composed of two heavy chains, each with a molecular weight of 50 kD, and two light chains, each with a molecular weight of 25 kD. IgM, which has two 65 kD heavy chains and two 25 kD light chains and exists as a pentamer, is a much larger molecule [25]. These immunoglobulin chains form distinct bands at their respective molecular weights upon SDS PAGE treatment. We have found the presence of bands at other locations after incubation with culture supernatant containing *P. levii* protease, showing that these protein chains have become fragmented. The results of our studies show that *P. levii* produces an enzyme which specifically cleaves IgG<sub>2</sub>, but not IgG<sub>1</sub> or IgM.

The description of a *P. levii* protease which is IgG specific is significant because such a protease may aid in the establishment of these bacteria during an infection by cleaving specific IgG which could potentially aid in clearance of the infection. In the bovine animal IgG<sub>2</sub> is the most important antibody in neutrophil-mediated phagocytosis [6] and in antibody dependent cell mediated cytotoxicity [27]. Antigen coated with IgG<sub>1</sub> are not readily adhered to by neutrophils or phagocytosed by these leukocytes. IgG<sub>2</sub> has also been shown to mediate phagocytosis by peripheral blood monocytes to a greater extent than IgG<sub>1</sub>, and IgM failed to do so without complement addition. Thus, the action of neutrophils is an important factor in the clearance of *P. levii* infection in the bovid. By producing a protease which specifically cleaves IgG<sub>2</sub> and inhibiting neutrophil-mediated phagocytosis, the organism increases its opportunities to create a viable infection in the bovine foot because the inability to successfully phagocytose infecting bacteria can compromise the host and aid in the progression of this infection. Thus, administration of an agent which neutralizes this protease will enhance neutrophil-mediated phagocytosis and speed resolution of the infection by the host.

The *P. levii* protease appears to function optimally at 35°C, a temperature not unlike that of the distal bovine limb. The specificity of this protease activity suggests that the enzyme may act specifically at the hinge region of the immunoglobulin. Such a region is not present in IgM and is the area with the highest degree of variance between IgG<sub>1</sub> and IgG<sub>2</sub> [28,29]. The near complete disappearance of heavy chain from the IgG preparation under conditions of lengthy incubation suggests that multiple enzyme activities are occurring and that the degradation of these Igs is complex.

In natural infections, neutrophils (cells that kill bacteria), but very few lymphocytes, are recruited to the area of infection. Under optimal circumstances, neutrophils can resolve the infection through phagocytosis (ingestion and destruction of the offending bacteria). It is well known that a Th<sub>1</sub> (T-helper lymphocyte 1) response and the associated IgG<sub>2</sub> that is produced by a Th<sub>1</sub> response, is critical for neutrophils to destroy bacteria by phagocytosis [6]. We have demonstrated that bacteria causing footrot (*Porphyromonas levii*) produce a specific toxin (a protease) that destroys only IgG<sub>2</sub> (see Figs. 1A through 1D). This toxin may act in the foot of affected cattle to

destroy the IgG<sub>2</sub> crucial for successful resolution of the infection by neutrophils. The use of this toxin in a vaccine composition will result in formation of antibodies which can neutralize or inhibit the toxin before it destroys Th<sub>1</sub> associated IgG<sub>2</sub>, thus allowing the neutrophils to phagocytize the bacteria.

5 Others [7] have postulated that immune responses can be altered and tailored to specific conditions (such as a Th<sub>1</sub> or a Th<sub>2</sub> response) through the strategic delivery of cytokines (immune messenger molecules). Such a tailored delivery of a vaccine may be useful in bovine footrot, where it would be useful to increase IgG<sub>2</sub> production to overcome the effects of the *Porphyromonas levii* protease toxin.

10 Bovine PMN efficiently phagocytosed *P. intermedia* and *F. necrophorum*, but not *P. levii*. High titre *anti-P. levii* serum increased the efficiency of phagocytosis of *P. levii* and this increase in phagocytosis was independent of heat labile complement components. *Porphyromonas levii* produced an Ig protease which cleaves bovine IgG, but not IgM. We postulate this specific humoral immunity may be involved in the  
15 resolution of this infection. *Porphyromonas levii* produces a protease that was found to cleave IgG<sub>2</sub>, the Ig class involved in PMN-mediated phagocytosis, but not IgG<sub>1</sub> or IgM. This enzyme is, thus, a significant virulence factor in AIP through local inhibition of immunoglobulin assisted PMN-mediated phagocytosis.

We have discovered in our PMN phagocytosis studies that the action of  
20 neutrophils is an important factor in the clearance of *P. levii* infection in the bovid affected with acute interdigital phlegmon. By producing a protease, or possibly several proteases, which act alone or collectively to specifically cleave IgG<sub>2</sub> and therefore inhibiting PMN-mediated phagocytosis, *P. levii* increases its opportunities to create a viable infection in the bovine foot. The inability to successfully phagocytose these  
25 bacteria could compromise the host and aid in the progression of this infection. Thus, agents which neutralize such protease are useful to treat footrot.

Studies from our laboratory show that PMN chemotactically move toward bacteria associated with acute footrot in an efficient manner, demonstrating that their motility is unaffected by these bacteria. Results are presented in Table A. Chemotaxis

is the movement of cells (here, PMN's) along a concentration gradient of the agent, while chemokinesis is the movement of PMN along a gradient of serum.

**Table A. Neutrophil Chemotaxis and Chemokinesis**

5	<b>Stimulus</b>	<b>Chemotaxis (<math>\mu\text{m/h}</math>)</b>	<b>Chemokinesis (<math>\mu\text{m/h}</math>)</b>
	Negative Control	11.2 + 0.5	94.1 + 5.6
	Positive Control	24.8 + 0.9	121.4 + 1.5
	<i>P. levii</i>	22.8 + 2.1	122.6 + 1.8
	<i>P. intermedia</i>	29.7 + 2.0	80.7 + 2.3
10	<i>F. necrophorum</i>	44.8 + 1.0	99.3 + 2.3
	All 3 Bacteria	32.1 + 2.3	110.7 + 2.8

Additionally, we have found that PMN demonstrate significant oxidative metabolism when exposed to *P. levii* or *F. necrophorum*, but that the percentage of PMN which are oxidatively metabolic decreased when exposed to *P. intermedia*. Thus, decreasing oxidative metabolism of PMN by *Prevotella intermedia* is likely an integral mechanism for these bacteria to evade host resolution of infection. Results are presented in Table B.

**Table B. PMN Oxidative Metabolism**

20	<b>Experimental Group</b>	<b>% PMN Oxidatively Active (Mean <math>\pm</math> SE) n=9</b>
	Resting	10.0 $\pm$ 0.6
	Stimulated (PMN)	94.0 $\pm$ 0.5
	<i>F. necrophorum</i>	80.8 $\pm$ 1.2
25	<i>P. levii</i>	82.8 $\pm$ 0.7
	<i>P. intermedia</i>	18.3 $\pm$ 0.7



*Prevotella intermedia* also produces a toxin (a  $\beta$ -lactamase enzyme) that destroys certain antibiotics, i.e., those which contain a  $\beta$ -lactam ring structure, such as penicillins and cephalosporins (Fig. 2). This enzyme has been partially purified by precipitating the enzyme in 40-50% ammonium sulfate (Fig. 3). Use of this enzyme in  
5 a vaccine composition will result in raising antibodies against the enzyme which inactivate its activity. This will allow more efficient use of antibiotics, even  $\beta$ -lactam antibiotics, to treat the infection.

We have shown via immunoblots with acute and convalescent sera of experimentally infected animals that one can successfully immunize cattle with antigens  
10 of *Porphyromonas* and *Prevotella* (Figs. 4A through 4C). Acute and convalescent sera from some feedlot steers with footrot show rising titers to these antigens (Fig. 5A through 5D), but the reaction is not as strong as that seen in experimentally infected steers. These Western blots indicate that antibodies are weakly or not at all produced against *Fusobacterium necrophorum*, further supporting a minimal role for this  
15 bacterium in the disease.

Moreover, histological assessment of experimentally infected feet indicates that predominantly macrophages (phagocytic and antigen presenting cells) and lymphocytes (antibody producing and processing cells that coordinate immunity) are present. These data show that *Porphyromonas* and *Prevotella* vaccine compositions provide effective  
20 humoral and cellular immune responses against footrot-causing organisms. Pre-exposure vaccination with vaccine compositions prepared from these organisms and/or their subunits and/or toxins provide antibody, immune memory cells, and other specific lymphocytes which will alter the course of infection through enhancing the function of neutrophils. The most effective treatment must negate the activities of the  
25 *Porphyromonas levii* IgG<sub>2</sub> toxin to allow neutrophil destruction of bacteria.

This invention provides vaccine compositions comprising the footrot causing bacteria *Prevotella* and *Porphyromonas*. It has been found, using surgical biopsy methodology to obtain specimens from bovine footrot, that these bacteria, and not *Fusobacterium necrophorum* which was traditionally thought to cause bovine footrot,  
30 are the causative agents of footrot. We have used these bacteria, grown under strictly

anaerobic conditions in the laboratory, in the presence of haemin and Vitamin K, to vaccinate animals. Further, we are able to produce and isolate bacterial toxins from *in vitro* cultures of these bacteria. These toxins can be used for protective immunization against infection by the bacteria causing bovine footrot or for raising toxin neutralizing antibodies for use in passive immunization of affected animals.

In particular, *Porphyromonas levii* and *Prevotella intermedia*, which are present in the cellulitic tissues of animals affected with bovine footrot, are preferred for use in the present invention. In a most preferred embodiment, the invention provides inactivated bacteria, toxins, and antibodies to toxins and antigens of *Porphyromonas levii* and *Prevotella intermedia*. Such inactivated bacteria, toxins, and antibodies are useful for preventing and treating bovine footrot and the symptoms thereof.

A variety of strains of *Porphyromonas* and *Prevotella* are useful in the present inventions. Strains which grow well *in vitro*, are able to infect the target animal species, and produce toxin when grown *in vitro*, are preferred. The bacterial strains used in this invention are cultured by growing them in appropriate media, e.g., bovine tissue based medium, such as chopped meat medium or brain heart infusion medium that has been supplemented with haemin and Vitamin K, with resazurin as an indicator, under conditions of strict anaerobiasis.

The invention also provides compositions comprising inactivated *Prevotella* and/or *Porphyromonas* bacteria which compositions are protectively immunogenic. Various strains of *Prevotella* and *Porphyromonas* bacteria may be useful in such vaccine compositions. Vaccine compositions may contain one or more of the bacteria causing bovine footrot, and/or one or more subunits and/or toxins of these bacteria. *Porphyromonas levii* and *Prevotella intermedia* are preferred. In particular, strains of *Porphyromonas levii* and *Prevotella intermedia* which produce large amounts of toxin when cultured *in vitro* are most preferred.

The bacterial strains may be cultured as set forth in the Examples below, then harvested for use in vaccine compositions. Bacteria are preferably inactivated before use in vaccine compositions. Various methods of disruption may be preferably used to inactivate the bacteria, including sonication, osmosis, use of pressure differentials or

freezing. Freezing is the most preferred. Conventional techniques such as mild heat treatment or formalin inactivation may also be used to inactivate bacteria or toxins used in vaccine compositions.

The formulation of such vaccine compositions may include suitable  
5 pharmaceutical carriers, including adjuvants. The use of an adjuvant, such as an alum-based adjuvant is preferred. Many commercial adjuvants may be useful in the present invention. For these studies an alum-based adjuvant containing aluminum hydroxide (Imject Alum, Rockford, IL) was used. Exact formulation of the vaccine compositions will depend on the particular formulation and the route of immunization. Such vaccine  
10 composition formulation is well-known to those skilled in the art [30].

Such vaccine compositions are useful for immunizing any animal susceptible to footrot. The present invention provides a method of preventing or treating footrot by administering an effective amount of a vaccine composition of *Prevotella* and/or  
*Porphyromonas* to an animal in need of such prevention or treatment. Bovines are  
15 preferable treated. In particular, preferred vaccine compositions comprise  
*Porphyromonas levii* and/or *Prevotella intermedia*.

The invention further provides compositions comprising neutralizing agents, such as antibodies, to *Prevotella* and/or *Porphyromonas* bacteria and/or their toxins or subunits, which compositions are useful to treat footrot. Formulation of such  
20 compositions may include suitable pharmaceutical carriers, and is also well-known to those skilled in the art.

The route of administration may be any convenient route, and may vary depending on the particular bacteria, subunit, toxin or agent used in the composition, the animal to be treated and/or vaccinated, and other factors known to those of skill in  
25 the art. Parenteral administration, such as subcutaneous, intramuscular, or intravenous administration is preferred. Subcutaneous administration is most preferred for food-producing cattle. Oral administration may be used, including oral dosage forms which may be enteric-release coated. Intraperitoneal, nasal and rectal routes of administration are also contemplated.

The schedule of administration may vary depending on factors such as the bacteria and the animal being treated. Animals may receive a single dose, they may receive an initial dose and a booster dose, or they may receive multiple doses. Annual boosters may be used for continued protection. In particular two doses 21 days apart  
5 are preferred as a primary course. Development of such schedules of administration is known to those of skill in the art.

The age of the animal to be treated may also affect the route and schedule of administration. For example, for vaccination, administration is preferred at an age when maternal antibodies are no longer present and the animal is immunologically  
10 competent. This is about 2-4 months of age in cattle. Additionally, administration of vaccine composition to mothers so that they develop antibodies is useful to prevent infection of their offspring through passive transfer of antibodies in colostrum and milk.

The methods of this invention are effective in preventing colonization of the subcutaneous tissues of the foot with bacteria which cause bovine footrot. They are  
15 also effective in preventing the symptoms of bovine footrot. This includes neutralization of toxin and prevention of the physiological effects of toxin which may occur when *Porphyromonas* and/or *Prevotella* are present in the foot of the animals.

Treatment may be administered to symptomatic or asymptomatic animals,  
20 including animals with chronic infection. Inducing the production of neutralizing antibody against *Porphyromonas* protease toxin or delivering antibody or another agent which neutralizes this toxin allows natural development of neutrophil associated antibody that provides successful recovery from infection with footrot causing organisms such as *Porphyromonas* and/or *Prevotella*. Such antibodies or other  
25 neutralizing agents will also allow development of antibodies to other organisms (such as *Fusobacterium necrophorum*) which may be associated with footrot.

The present invention also provides toxins of *Porphyromonas levii* and *Prevotella intermedia* and methods of producing these toxins. It has been found that these organisms, when cultured in medium with haemin and Vitamin K under anaerobic  
30 conditions, produce certain toxins *in vitro*. These toxins may be used to immunize

animals against footrot. The toxins may be isolated from the supernatants of the bacterial cultures. They are useful for preventing and treating infection.

Supernatants from the cultured bacteria (*Porphyromonas levii*) contain a toxin (protease) that specifically cleaves bovine IgG<sub>2</sub> into fragments. Isolated toxin was incubated with biotin-labeled immunoglobulin and the resulting mixture separated by SDS PAGE. When active toxin was present, IgG<sub>2</sub> fragments were produced as the toxin cleaved the immunoglobulin. Immunoglobulins remained intact when no toxin was present or when the toxin was purposely inactivated. The toxin was also shown not to be active against other immunoglobulins (i.e., IgG<sub>1</sub> and IgM). Figs. 1A through 1D present these results.

Supernatants from the cultured bacteria (*Prevotella intermedia*) contain a toxin ( $\beta$ -lactamase enzyme) that specifically cleaves  $\beta$ -lactam antibiotics. See Fig. 2. This toxin has been purified using ammonium sulfate precipitation methods.

The present invention also provides antibodies to *Prevotella* and *Porphyromonas* and/or their toxins or subunits. Polyclonal and monoclonal antibodies may be raised by conventional techniques. These antibodies will be useful to enhance neutrophil-mediated phagocytosis and as an antiserum to neutralize the effects of toxin. Thus, when administered to an animal, e.g., intravenously, they may be expected to relieve symptoms of bovine footrot.

Two strains of anaerobic bacteria, *Porphyromonas levii* and *Prevotella intermedia*, were assessed for their ability to induce acute footrot in cattle. They were used in conjunction with *Fusobacterium necrophorum*. Cattle feet were experimentally infected with these bacteria and animals were examined clinically, histopathologically, and bacteriologically for response to inoculation with these anaerobic bacterial species. After experimental induction of footrot, response to a macrolide antibiotic to evaluate its potential usefulness as a footrot treatment was examined. *Porphyromonas levii* caused a significantly more severe infection than *Prevotella intermedia*, as assessed by clinical scoring, when inoculated into the bovine interdigital space. Histological examination of affected tissue demonstrated local edema, necrosis, infiltration of the subcutaneous tissue with leukocytes, and bacterial cells morphotypic of the strains used

to infect the interdigital tissues. In all cases bacteria recovered from biopsy samples of the infected feet six days following experimental infection were consistent with those strains initially injected into the animals. MIC's and MBC's of bacteria used to infect feet were not different from the MIC's and MBC's of bacteria recovered from the feet  
5 six days later, but prior to therapy. All animals were treated with antibiotic on the sixth day following the experimental infection and 83.3% (5/6) responded very favorably to this treatment, based on a subjective assessment of the severity of lameness, swelling, and lesions.

Cattle injected in the interdigital skin and subcutaneous tissue with  
10 *Porphyromonas levii* or *Prevotella intermedia*, together with *Fusobacterium necrophorum*, developed a clinical condition consistent with acute footrot within five days, causing interdigital necrosis, swelling of the interdigital tissues and distal subcutaneous tissue, and lameness. This experimental model resulted in lesions and lameness typical of the natural infection in cattle. Histopathological features (edema,  
15 leukocyte infiltration, necrosis) in the subcutaneous tissues were also typical of natural infection. All of these findings are considered characteristic of acute footrot. We have demonstrated that *Porphyromonas levii* or *Prevotella intermedia* inoculated into the interdigital skin of cattle can induce acute footrot, in particular when injected in conjunction with *Fusobacterium necrophorum*.

20 This experimental model infection system is useful to further examine the pathophysiological and immunological consequences of acute bovine footrot and is a useful screening tool for the evaluation of potential new therapeutics for footrot, such as antibiotics and vaccines. Based on these results, it appears that the macrolide antibiotic we tested is a useful antibiotic for the treatment of acute bovine footrot.

25

### C. Examples of Embodiments of the Invention

The following examples are not intended to limit the scope of the invention in any manner. In general the following materials and methods were used in the examples unless otherwise noted:

30

# 1. Medium Preparation.

The medium used for culture of the *Porphyromonas levii* and *Prevotella intermedia* (Brain Heart Infusion broth with haemin and Vitamin K and resazurin) was prepared as indicated below.

5	<u>Medium Component</u>	<u>g/l</u>
	Calf brains, infusion from	200 g
	Beef heart, infusion from	250 g
	Bacto protease peptone	10 g
	Bacto dextrose	2 g
10	Sodium chloride	5 g
	Disodium phosphate	2.5 g
	Hemin	5 mg
	Vitamin K	1 mg
	Resazurin	1 mg

15

# 2. Biopsy Isolation of *Porphyromonas levii* and *Prevotella intermedia*.

The foot of a bovine with clinical footrot was lifted and cleaned using water and disinfectant solution followed by 70:30 (vol/vol) ethanol:water. A small incision was made using a scalpel blade (#10) over a swollen portion of the limb immediately proximal to the affected region of the foot. A Trucut™ hepatic biopsy needle was inserted into the subcutaneous tissue. A core of tissue was collected, placed in pre-reduced transport medium (Anaerobe Systems, San Jose, CA) and transported immediately to the laboratory in anaerobic transport medium.

*Porphyromonas levii* and *Prevotella intermedia* were grown separately on kanamycin:vancomycin laked blood agar and Brucella blood agar for five days under conditions of 5% H<sub>2</sub>:5% CO<sub>2</sub>:90% N<sub>2</sub> at 37°C. A single colony of each organism was inoculated into a separate tube of modified chopped meat medium and grown for 24h at 37°C in an anaerobic chamber.

30

3. Freezing *Porphyromonas levii* and *Prevotella intermedia*.

The isolated bacteria were kept frozen at -85°C in either pre-reduced transport medium or semisolid BHI broth under anaerobic conditions.

5 4. Growing *Porphyromonas levii* and *Prevotella intermedia*.

Both bacterial species were grown in supplemental BHI broth (above). Broths were inoculated from secondary cultures that have been expanded from primary cultures stored in the laboratory. Bacteria were incubated at 37°C in an anaerobic atmosphere (5% carbon dioxide, 5% hydrogen, balance nitrogen). Broths of  
10 *Porphyromonas levii* were incubated 96h and *Prevotella intermedia* broths were incubated 72h.

5. Harvesting *Porphyromonas levii* and *Prevotella intermedia*.

Bacteria were harvested by centrifugation at 3000 x g for 10 min. Pellets were  
15 resuspended and washed three times in sterile PBS.

6. Preparing Vaccine from *Porphyromonas levii* and *Prevotella intermedia*.

To lyse cells the washed bacteria were snap frozen in liquid nitrogen, thawed, and snap frozen again. Protein concentration was determined by the Bradford protein  
20 assay and vaccines were stored at -20°C until needed.

### EXAMPLE 1

#### Immunological Assessment of Animals Naturally Infected with Bovine Footrot

25 To assess the humoral immunity (antibody production) resulting from natural infection with bovine footrot, acute and convalescent sera were obtained from affected animals. Acute serum was collected via jugular venipuncture on the day an animal initially showed clinical signs of acute bovine footrot (i.e., lameness, swelling of the interdigital region and distal foot, and/or a necrotic lesion of the interdigital space).  
30 Twenty-eight days following appearance of clinical signs, a second (convalescent)



sample was collected, also by jugular venipuncture. Serum was separated from cells and stored at -85°C until used in the Western blotting assays.

*Porphyromonas levii* (7-5) was grown on Brucella blood agar and inoculated into pre-reduced chopped meat medium with Vitamin K, hemin and resazurin and  
5 grown anaerobically (5% H<sub>2</sub>:5%CO<sub>2</sub>:90%N<sub>2</sub>) for 24h. *Prevotella intermedia* (5-2) was grown under similar conditions for 48h and *Fusobacterium necrophorum* ATCC 27852 (ovine footrot isolate) was grown for five days on chocolate agar under similar conditions, inoculated into modified chopped meat medium and grown anaerobically for 24h. Bacteria were harvested by centrifugation (5000 x g for 15 min) and the pellets  
10 placed in frozen aliquots. Protein concentrations of these aliquots of centrifuged bacterial cells following a freeze-thaw were determined by the Bradford protein assay.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was conducted on these bacterial specimens (10 µg protein per lane) following boiling in SDS PAGE reducing and denaturing sample buffer according to accepted methodology  
15 [31]. Electrophoretically separated proteins within the gel (4% stacking gel and 12% resolving gel) were electrophoretically transferred to nitrocellulose paper (18h at 4°C and 30V) according to accepted methodology [32]. Protein transfer was confirmed by amido black staining of a strip of nitrocellulose paper. Strips were blocked with 1% skim milk in Tris buffered saline and exposed to either acute serum or convalescent  
20 serum from cattle with footrot. Sera were used at the same dilution (1:200). A monoclonal antibody raised against bovine IgG (heavy chain specific) conjugated to alkaline phosphatase was used as a detection method for bound bovine IgG. Color reaction involved incubation of nitrocellulose strips with nitro blue tetrazolium in 0.2M Tris-HCl 4mM MgCl<sub>2</sub> pH 8.8 buffer with 5-bromo-4-chloro-3-indolyl phosphate in  
25 dimethyl sulfoxide. Development of blots proceeded for exactly 10 min and the development reaction was stopped with ice water. Blots were immediately photographed.

Western blotting results from four animals are shown in Figs. 5A through 5D. In each figure, the acute (A) and convalescent (C) serum reactions are shown against  
30 the antigens from *Fusobacterium necrophorum* ATCC 27852, *Prevotella intermedia*

(5.2) and *Porphyromonas levii* (7.5). In animal 4203OR (Fig. 5A), increased titers of IgG antibodies against *Prevotella intermedia* (5.2) and *Porphyromonas levii* (7.5) were seen in convalescent serum relative to acute serum. Only a slight increase in antibody titer was seen with *Fusobacterium necrophorum* ATCC 27852. In animals 1875RED (Fig. 5B), 2281GRN (Fig. 5C), and PUR01 (Fig. 5D), no rising titers of IgG against *Fusobacterium necrophorum* were detected, but increasing IgG titers to one or both of *Prevotella intermedia* or *Porphyromonas levii* were clearly observed. This is evidenced by the increasing number and intensity of the protein bands observed on the strips in the convalescent sera samples. These data show that over the 28-day period in which the animals recovered from the disease, a specific immune response was produced against *Prevotella intermedia* (5.2) and/or *Porphyromonas levii* (7.5), but no substantive production of IgG specifically reactive to *Fusobacterium necrophorum* was detected. These data indicate *Fusobacterium necrophorum* is not immunologically recognized by the host. They also indicate that *Prevotella intermedia* and/or *Porphyromonas levii* are important agents in the disease, and that a comparatively weak (1:200 dilutions) serum IgG response is produced toward these bacteria in the course of natural infection.

## EXAMPLE 2

### Immunological Assessment of Animals Experimentally Infected with Bovine Footrot

To assess the humoral immunity (antibody production) resulting from experimental infection with bovine footrot, pre-exposure serum, acute serum, and convalescent sera were obtained from animals experimentally infected with bovine footrot as described in Example 5 below. Pre-exposure serum was collected prior to infection by jugular venipuncture. Acute serum was collected by jugular venipuncture on the day following experimental infection and a second (convalescent) sample was collected, also by jugular venipuncture, 11 days following infection. Serum was separated from cells and stored at -85°C until used in the Western blotting assays.

*Porphyromonas levii* (7-5), *Prevotella intermedia* (5-2) and *Fusobacterium necrophorum* ATCC 27852 (ovine footrot isolate) proteins were prepared as described in Example 1.

Western blotting results (performed as described in Example 1) from a steer  
5 experimentally infected with *Porphyromonas levii* and *Fusobacterium necrophorum* are shown in Figs. 4A through 4C. In Fig. 4A the antigen used was *Fusobacterium necrophorum* ATCC 27852. The results shown in Fig. 4A clearly demonstrate that levels of IgG against *F. necrophorum* are negligible at all time periods tested—pre-exposure, acute exposure and convalescent exposure. Conversely, this animal showed  
10 rising IgG titers to both *Prevotella intermedia* 5.2 (Fig. 4B) and *Porphyromonas levii* 7.5 (Fig. 4C), indicating some shared antigenicity between *Prevotella intermedia* 5.2 and *Porphyromonas levii* 7.5. The negligible immune response to *Fusobacterium necrophorum* suggests that it is not readily recognized by the host immune system and/or that it is not involved in the active infection of the subcutaneous tissues in  
15 footrot.

Four of the six experimentally infected animals were re-assessed as above approximately eight weeks following experimental infection to determine the presence of serum IgG specific for whole bacterial cell extract antigens. The pattern of serum IgG remained the same. This demonstrates that the serum IgG produced in response to  
20 experimental infection lasts for a minimum of several weeks and similar lasting serum IgG may be created via vaccination. The data show that, despite the known presence of *Fusobacterium necrophorum* within experimentally infected tissue, no substantial immunological response to this bacterium occurs in experimentally infected cattle, while *Prevotella intermedia* and/or *Porphyromonas levii* are the important etiologies of  
25 the disease.

### EXAMPLE 3

#### Production and Assessment of Immunoglobulin G2 Destroying Toxin

N-hydroxy-succinimidobiotin (Sigma Biosciences, St. Louis, MO) was  
30 dissolved in dimethyl sulfoxide to a 1 mg/ml concentration. It was then added to equal

one tenth the weight of bovine IgG (Sigma Biosciences, St. Louis, MO), IgM (Sigma Biosciences, St. Louis, MO), IgG<sub>1</sub> (Chemicon International, Inc., Temecula, CA) and IgG<sub>2</sub> (Chemicon International, Inc., Temecula, CA). The solution was then incubated for 2h at 25°C and the reaction stopped with 1 mg of glycine (Sigma Biosciences, St. Louis, MO). The biotinylated immunoglobulins were dialyzed overnight in three changes of double distilled water. Final concentrations were 10 mg/ml for IgG, IgG<sub>1</sub> and IgG<sub>2</sub> and 1.1 mg/ml for IgM.

Supernatant of a broth culture of *P. levii* grown in anaerobic conditions (5% H<sub>2</sub>:5% CO<sub>2</sub>:90% N<sub>2</sub>) in Cooked Meat broth (BBL, Cockysville, MD) (supplemented with 5 mg/l of hemin, 1 mg/l of Vitamin K and 1 mg/l resazurin (Sigma Biosciences, St. Louis, MO) at 37°C for four days was collected by centrifugation. The bacterial culture was centrifuged at 3020 x g for 20 min to remove bacterial cells. The remaining supernatant was placed in aliquots of 250 µl amounts for the protease assay. Culture purity was assured by streak plating onto Brucella Blood Agar and standard bacteriological identification.

Procedures were slight modifications of the methods of Lee and Shewen [24]. Ten microliters of biotinylated immunoglobulin was added to 250 µl of bacterial culture supernatant and 2.5 µl of penicillin-streptomycin solution. The mixture was incubated at 35°C and 10 µl samples taken at 0, 0.5, 1, 2, 4, 8, 24, 48, and 72h. The samples were added to a 10 µl of sample buffer, boiled for 4 min and analyzed by SDS PAGE, electrophoretic transfer and streptavidin-alkaline phosphatase exposure. Controls included *P. levii* supernatant heated to 100°C prior to exposure to the biotinylated Igs and uninoculated culture supernatant.

All samples were loaded onto 15% resolving gels with 4% stacking gels for SDS PAGE using standard methodology [31]. Gels were run on ice at 150V for 15 min and at 200V for 45 min in 10% running buffer. The proteins in the gel were then transferred to nitrocellulose paper using standard methods [32]. Transfer was at 20V overnight at 4°C. Blocking was conducted using a 1h incubation at 37°C in a solution of skim milk powder (2% wt/vol) and Tris buffered saline containing Tween 20 (18g NaCl, 2.42g Trizma base, 2 l double distilled water, 1 ml Tween 20, pH 7.4) (TBS-

tween). Blots were washed with TBS-tween three times (15 min) at room temperature, then incubated in a 1:5000 dilution of streptavidin-alkaline phosphatase (Sigma Biosciences, St. Louis, MO) in TBS-tween for 2h at 37°C. The blots were again washed three times for 15 min in TBS-tween. Nitroblue tetrazolium (Sigma Biosciences, St. Louis, MO) (NBT) and 5-bromo-4 chloro- 3 indolyl phosphate (Sigma Biosciences, St. Louis, MO) (BCIP) were added as substrate for color development, which was allowed to proceed for 4 min. Substrate solution was composed of 10 mg of NBT dissolved in 100 ml of developing buffer (31.52g Tris-HCL, 0.18g  $MgCl_2 \cdot 6H_2O$ , 1 l distilled water, pH 8.8) and 5 mg of BCIP dissolved in 1 ml of dimethyl sulfoxide was added to this solution. Protein bands representing the light and heavy chains of the biotinylated immunoglobulins, or fragments of these immunoglobulin chains were viewed based on alkaline phosphatase activity on the nitrocellulose paper. Photographs were obtained immediately.

Assays performed on biotinylated IgG produced degradation bands, fragments of the immunoglobulin heavy and light chains, by 0.5h, as seen in Fig. 1A. The intensity of these Ig fragment bands continued to increase during the 72h of incubation. Optimization studies performed on IgG showed that incubation of the supernatant with IgG at 35°C showed the most pronounced activity and that a streptavidin-alkaline phosphate dilution of 1:5000 gave optimal results. All further assays were performed under these same conditions. When IgM was examined using these methods (Fig. 1B) there were no degradation bands (immunoglobulin fragments) formed. Only bands representing intact heavy and light chains of IgM could be visualized. When  $IgG_1$  was assayed, degradation bands were not evident (Fig. 1C) and again only bands representing intact heavy and light immunoglobulin chains could be recognized. However, when assays using  $IgG_2$  were conducted results similar to those performed with mixed subtype IgG were seen. Degradation bands were evident within 30 min, as demonstrated in Fig. 1D. Controls using heated supernatant and uninoculated media, each of which was exposed to the biotinylated bovine IgG, showed no evidence of degradation bands. This indicated that fragmentation of the immunoglobulins was the result of *Porphyromonas levii* enzyme activity.

#### EXAMPLE 4

##### Production and Assessment of $\beta$ -Lactamase Enzyme Toxin

The major mechanism of resistance to penicillin and other  $\beta$ -lactam antibiotics is the presence of a group of bacterial enzymes termed  $\beta$ -lactamases. Since their first  
5 discovery in the 1940's [33], the widespread use of penicillins is thought to have led to an increased occurrence of  $\beta$ -lactamases [34].  $\beta$ -lactamases are enzymes capable of hydrolyzing  $\beta$ -lactam antibiotics such as the penicillins and the cephalosporins. They can be chromosomally mediated or plasmid mediated and constitutive or inducible [35].  $\beta$ -lactamase inhibitors, such as clavulanic acid or sulbactam, can be used to prevent the  
10  $\beta$ -lactamase from inactivating the  $\beta$ -lactam antibiotic, but these inhibitors have slightly different inhibition profiles [36], so the choice of which inhibitor to use will depend on the specific type of  $\beta$ -lactamase present. Specific immunity raised against the enzyme may allow more successful therapy.

Antibiotic Susceptibility Tests: Four anaerobic bacterial isolates of *Prevotella*  
15 *intermedia* were retrieved from storage in Amies medium at  $-85^{\circ}\text{C}$  and plated onto Brucella blood agar (BBA) plates. Penicillin resistance was tested using a disc diffusion method. Colonies from BBA plates were suspended in vials of sterile saline (0.85% NaCl) to reach a turbidity roughly equal to tube 3 of the McFarland turbidity standards (approximate equivalent density of  $9.0 \times 10^8$  bacteria/mL). One (1) mL of  
20 that suspension was transferred to a fresh BBA plate and spread equally around the plate to create a lawn of bacteria. The plate was allowed to dry before applying two antibiotic discs to the surface of the agar; one disc contained 2 units of penicillin G (P2) and the other contained 10 units (P10). The plate was incubated overnight in an anaerobic chamber at  $37^{\circ}\text{C}$  and the diameters of any zones of inhibition were  
25 measured.

$\beta$ -Lactamase Testing: Any of the above bacterial isolates showing resistance to penicillin G were tested for  $\beta$ -lactamase activity using commercially available nitrocefin impregnated filter paper discs (Cefinase - BBL). Some results using assay are demonstrated in Fig. 2. Colonies from the antibiotic susceptibility plates and from  
30 normal BBA plates were tested to determine if the  $\beta$ -lactamase activity was induced by

antibiotic exposure. Colonies removed from an agar plate were smeared onto the disc surface and the disc was observed for a color change for up to 1h. If the disc changed from yellow to red in color, it was considered to be a positive result, while no color change indicated a negative result. Note that for testing broths or other liquids, a drop  
5 of the fluid to be tested was added to the dry disc and then observed for a color change.

Enzyme Isolation: The *Prevotella intermedia* isolates exhibiting  $\beta$ -lactamase activity were used to isolate the  $\beta$ -lactamase itself. As previously outlined, the bacteria of interest were grown in Brain Heart Infusion broth cultures supplemented with 0.005% hemin and 0.5% yeast extract. Three hundred fifty (350) mL broth cultures  
10 were made anaerobic by bubbling nitrogen gas through them before autoclaving with tightly sealed caps. The cultures were inoculated, then left to incubate at 37°C in an anaerobic chamber for 48h. Following incubation, the broth contents were tested for  $\beta$ -lactamase activity, and a BBA plate was made to check the purity of the culture. The cultures were centrifuged at 5,000 x g for 15 min at 4°C. The supernatant was  
15 discarded and the pellets resuspended in 50 mM phosphate buffer, pH 7 and centrifuged at 15,000 x g for 40 min at 4°C. This washing procedure was repeated once more and the resulting pellet (resuspended in 50 mM phosphate buffer) was sonicated at 60% maximum with 6-7 bursts of 30 seconds each, to disrupt the cells. The sonicate was centrifuged at 27,000 x g for 1h at 4°C and the supernatant retained for ammonium  
20 sulfate precipitation.  $\beta$ -lactamase activity was periodically re-checked throughout the enzyme preparation.

Ammonium Sulfate Precipitation: The supernatant from the above procedure was subjected to ammonium sulfate precipitation [37] to begin the purification process. Sufficient ammonium sulfate was initially added to achieve 40% saturation and the  
25 mixture was stirred on ice for 20 min. This was then centrifuged at 10,000 x g for 20 min at 4°C. The pellet was resuspended in 50 mM phosphate buffer and saved as the 40% saturation fraction, while the supernatant was used further in the precipitation. Taking into account how much ammonium sulfate had already been added, enough ammonium sulfate was added to bring the supernatant to 50% saturation. This was  
30 stirred over ice for 20 min then centrifuged at 10,000 x g for 20 min at 4°C. The pellet

was resuspended and saved as the 40-50% saturation fraction. The procedure was repeated once more for 60% saturation, after which the resuspended pellet was saved at the 50-60% fraction and the supernatant was saved as the greater than 60% fraction. The ammonium sulfate was removed from the four fractions by overnight dialysis using  
 5 dialysis tubing with a molecular weight cut-off of 25,000 Daltons in a flask with distilled water constantly flowing through. The samples were then tested for  $\beta$ -lactamase activity and those fractions testing positive were used as enzyme isolates.

Gel Electrophoresis: The isolated enzyme fraction was run on a 7% SDS PAGE, and then stained with silver stain to visualize proteins in the sample.

10 Antibiotic susceptibility tests were performed on the bacterial isolates. Results are shown in Table 1. *Prevotella intermedia* isolates 5-2, 8-2 and 14-7 showed zones of what has been called regrowth. There was indeed a zone of inhibition, but within that zone of inhibition, there was re-growth immediately around the antibiotic disc. *Bacteroides fragilis* (7-6) was clearly resistant to penicillin.

15

**Table 1. Zones of Inhibition of Isolates from  
Bovine Footrot Tissue Samples**

	Isolate Identification	P2 zone of inhibition (mm)	P10 zone of inhibition (mm)
20	<i>Prevotella intermedia</i> (5-2)	22 (with regrowth)	28 (with regrowth)
	<i>Bacteroides fragilis</i> (7-6)	0	0
	<i>Prevotella intermedia</i> (8-2)	27 (with regrowth)	35 (with regrowth)
25	<i>Prevotella intermedia</i> (14-7)	34 (with 22mm regrowth)	38 (with 23mm regrowth)

$\beta$ -Lactamase Testing results are shown in Table 2, and demonstrate that *Prevotella intermedia* isolates 5-2, 8-2 and 14-7 produced  $\beta$ -lactamase.

30



**Table 2.  $\beta$ -lactamase Test Results from Footrot Isolates  
Grown on Two Media Types**

	<b>Isolate Identification</b>	<b>Antibiotic Susceptibility Plate</b>	<b>BBA Plate</b>
5	<i>Prevotella intermedia</i> (5-2)	positive	positive
	<i>Bacteroides fragilis</i> (7-6)	strongly positive	strongly positive
	<i>Prevotella intermedia</i> (8-2)	positive	positive
10	<i>Prevotella intermedia</i> (14-7)	positive	positive

Isolates of *Porphyromonas levii* were found to be sensitive to the antibiotic penicillin G and were  $\beta$ -lactamase negative.

15 Enzyme activity of the ammonium sulfate precipitation fractions from strain 7-6 (*B. fragilis*) was found in the 50-60% fraction, but with 5-2 (*P. intermedia*) the enzyme was found in the 40-50% fraction.

Results of gel electrophoresis are illustrated in Fig. 3. These fractions contained enzyme activity, although the activity has not yet been attributed to any single  
20 protein band within the fractions.

## **EXAMPLE 5**

### **Experimental Infection Model**

Experimental Animals: Yearling Canadian Simmental crossbred steers (400 kg)  
25 were used for this investigation (n=6). Each animal was individually identified with an eartag and commingled in a large pen. Steers were fed a diet of good quality hay ad libitum and fresh water. Animals were obtained from a single source and held for seven days prior to the initiation of the study. Animals were examined by a veterinarian to ensure suitability for the study. All procedures were conducted  
30 according to the guidelines of the Canadian Council on Animal Care.

Growth of Bacteria: *Porphyromonas levii* and *Prevotella intermedia*, both of which were isolated from the subcutaneous tissues of feedlot animals affected with acute footrot, were grown separately on kanamycin:vancomycin laked blood agar for five days under conditions of 5% H<sub>2</sub>:5% CO<sub>2</sub>:90% N<sub>2</sub> at 37°C. A single colony of each  
5 organism was inoculated into modified chopped meat medium (BBL) and grown for 24h at 37°C in an anaerobic chamber. *Fusobacterium necrophorum* (ATCC 27852) was grown anaerobically for five days on chocolate agar, and a single colony was inoculated into modified chopped meat medium and grown under anaerobic conditions for 24h, as described above. Samples of washed cells from broth culture were serially diluted and  
10 spread on agar to determine the precise number of viable bacteria (CFU/ml) in each suspension of bacteria. All manipulations were conducted under anaerobic conditions and these bacteria were not exposed to air until immediately prior to injection into the interdigital region of the steers.

Experimental Infection: The animals were individually restrained in a head gate  
15 and squeeze apparatus and the left hind foot lifted and restrained with rope. The interdigital region of the foot was carefully cleaned with water and a disinfectant solution and rinsed with 70% ethanol:water (vol:vol). Local anaesthesia was employed using lidocaine and the interdigital skin was excoriated using mechanical abrasion. *Porphyromonas levii* and *Fusobacterium necrophorum*, or *Prevotella intermedia* and  
20 *Fusobacterium necrophorum*, were drawn into a common syringe. This mixture (1.0 ml), containing 10<sup>9</sup> of each of the two organisms, was injected into the interdigital skin and subcutaneous tissue (0.5 ml per location). Three steers received *Porphyromonas levii* mixed with *Fusobacterium necrophorum* (Group 1) and three steers received *Prevotella intermedia* mixed with *Fusobacterium necrophorum* (Group 2). The animals  
25 were monitored closely after experimental inoculation for any immediate adverse reactions and daily thereafter.

Clinical Scoring: The severity of clinical signs present in each animal following experimental infection was monitored and scored using a slight modification of a previously published scoring system [17]. Briefly, this involved daily examination of  
30 the animal and subjective assessment of the lameness, lesion, and swelling.

Lameness was evaluated daily as:

- 0 = normal
- 1 = slight lameness - puts some weight on foot but moves readily
- 2 = moderate lameness - does not want to put weight on foot and moves  
5 slowly
- 3 = severe lameness - holds foot up at intervals and is reluctant to move or  
place weight on the foot (prefers to lie down)

Swelling was evaluated and scored daily as:

- 10 0 = no swelling
- 1 = slight to moderate swelling
- 2 = moderate to severe swelling
- 3 = severe swelling

15 Lesions were evaluated and scored daily as:

- 0 = no lesions
  - 1 = lesion healed or healing
  - 2 = small interdigital lesion extending up to  $\frac{1}{4}$  to  $\frac{1}{2}$  length of the  
interdigital space
  - 20 3 = very large necrotic lesion involving almost all of the interdigital space
- Scores for lameness, swelling, and lesions were totaled to arrive at a footrot score.

Biopsy Sampling of Subcutaneous Tissue: Tissue biopsy specimens were obtained from each experimentally infected animal six days after inoculation with  
25 bacteria, and immediately prior to antibiotic therapy. Each animal had its affected foot physically restrained using ropes. The foot was lifted and cleaned using water and disinfectant solution followed by 70:30 (vol/vol) ethanol:water. Local anaesthesia was applied and a small incision was made using a scalpel blade (#10) over a swollen  
portion of the limb immediately proximal to the affected region of the foot. A Trucut™  
30 hepatic biopsy needle was inserted into the subcutaneous tissue. A core of tissue was

collected and transported immediately to the laboratory in anaerobic transport medium. A second biopsy specimen was collected from each animal and fixed in 10% neutral buffered formalin for histology.

Bacteriology: Tissue samples were placed in modified chopped meat medium and incubated at 37°C under anaerobic conditions. Periodically, a small sample of broth was removed from the broth culture and streaked on kanamycin:vancomycin laked blood agar, Brucella agar, and on chocolate agar to obtain single colonies of the anaerobic bacteria present. Isolates were presumptively identified based on colony morphology and pigmentation, fluorescence under longwave UV light, gram stain reaction, cellular morphology, antibiotic resistance (kanamycin, vancomycin, colistin), catalase production, nitrate test, indole test, lipase production on egg yolk agar, and the urease and oxidase tests.

Antibiotic Sensitivity of Bacterial Isolates: Bacteria were grown anaerobically on kanamycin:vancomycin laked blood agar for colony isolation and purity. Isolates were then inoculated into 10 ml of modified chopped meat medium and incubated anaerobically at 37°C in an environmental chamber. Oxytetracycline hydrochloride and a macrolide antibiotic were diluted in medium to a concentration of 640 µg/ml. Serial dilutions (1:1) were made for both compounds to yield stock solution ranging from 640 µg/ml to 0.0625 µg/ml. Falcon 3072 Micro Test III™ tissue culture plates with lids were loaded in triplicate with 100 µl of each concentrations (MIC). Each drug concentration was inoculated with 100 µl of bacterial broth inoculum. The inoculum diluted 1:1 in sterile PBS (pH 7.2) served as a control. Drug stock solution was also aliquoted to measure absorbance capabilities (650 nm). Plates were incubated for 24h anaerobically at 37°C. The broth inoculum was serially diluted, plated on kanamycin:vancomycin laked blood agar and incubated anaerobically for viable counts (CFU/ml). After the incubation period, 20 µl of inoculum-drug combination was removed from one series of each descending drug concentration and plated on kanamycin:vancomycin laked blood agar to determine minimum bactericidal concentration (MBC) values. All MBC plates were incubated for seven days under anaerobic conditions. The tissue culture plates were then read on a microplate reader

using SOFTmax Version 2.32 software. Mean absorbance (650 nm) readings and grey scale analysis were used to determine the MIC for each drug and isolate tested. The MBC was determined by the drug concentration which exhibited a 99.9% reduction of CFU/ml count.

- 5 All six steers were normal and healthy prior to the study. No concurrent disease occurred during the period of this study. All six steers developed a clinical condition consistent with acute footrot within five days following inoculation of the bacteria into the subcutaneous tissue and the skin of the interdigital cleft. Each of the six animals was also clinically lame by five days post-inoculation with bacteria.
- 10 Biopsy specimens were successfully obtained from all animals on the sixth day following experimental infection. In each case anaerobic bacteriology produced the re-isolation of the organisms initially injected into the feet. Microscopic lesions were very similar in all steers. Typical lesions involved coagulative necrosis and edema of the subcutaneous tissues and an infiltration of local tissue with monocytic inflammatory
- 15 cells. Very few neutrophils were seen; however, biopsy samples were taken six days after exposure to the bacteria. An incubation period of five days after inoculation with bacteria may suggest that an initial failed neutrophilic response followed by a more chronic monocytic infiltration may precede clinical signs in this model infection. The implications of this finding in natural infections of footrot are not known. Morphotypic
- 20 bacteria were visible in stained sections of the biopsy specimens.

#### **EXAMPLE 6**

##### **Assessment of the Effectiveness of a Macrolide Antibiotic to Treat Footrot**

- Treatment: Six days following experimental inoculation of feet with bacteria,
- 25 and immediately following biopsy specimen collection, all steers from Example 5 were treated with a single subcutaneous administration of a macrolide antibiotic at a dosage of 10 mg per kilogram body weight. Response to the therapy was monitored by clinical scoring, as outlined above. Twenty-four (24) hours following administration of the antibiotic, the feet were prepared for biopsy sampling of subcutaneous tissue and a third

biopsy taken, held on dry ice for transportation to the laboratory, frozen in N<sub>2(l)</sub>, and stored at -85°C.

Histology: Tissue specimens fixed in 10% neutral buffered formalin were dehydrated in solutions of increasing ethanol concentration followed by three 15 min exposures to absolute ethanol. The specimens were then infiltrated with and embedded in meta-methacrylate. Sections (1.5 µm) were cut on a microtome, heat fixed on microscope slides, and stained with buffered methylene blue and basic fuchsin [19]. Coverslips were fixed on slides with mounting medium and sections were viewed and photographed on a microscope.

Statistical Analysis: Clinical scores were evaluated by comparison to pre-exposure scores in the same groups of animals using nonparametric analysis of variance and Kruskal Wallis tests. Data of Group 1 and Group 2 were compared by nonparametric analysis of variance and Mann Whitney tests. In all cases the P value was 0.05. The mean total footrot scores for animals in Group 1 and Group 2 are shown in Fig. 6A and Fig. 6B, respectively. In the *Porphyromonas levii* infected animals (Group 1) the mean total footrot score from day 7 was significantly reduced following antibiotic therapy on day 6. Scores remained significantly lower than on day 6 and continued to decrease from day 8 to day 17. In the *Prevotella intermedia* infected animals (Group 2) there was a reduction in clinical score; however, the reduction was not statistically significant. Fig. 7 demonstrates the mean total footrot scores from all six animals (irrespective of group). There was significant improvement in the footrot score in five of the six animals following treatment and this reduction in clinical score was maintained for the entire 17 days of the experiment. The single failure (animal number YEL398) was initially the most severely affected animal following experimental infection. It did not respond to a single administration of the macrolide antibiotic, or to a second administration of the drug 48h later. Treatment with ceftiofur sodium at a dosage of 1.0 mg ceftiofur per kg body weight q24h for five days was implemented following the initial treatment course. The steer retained a subtle and permanent lameness and was eventually euthanized 30 days following the last treatment and given a necropsy examination. The post mortem examination revealed fibrosis of

the lateral branch of the long digital extensor tendon. There was no evidence of active infection grossly or histologically. Fig. 8 shows the mean total footrot scores of animals excluding the non-responding YEL398.

- 5 Table 3 demonstrates the minimum inhibitory concentrations and minimum bactericidal concentrations of the three strains of bacteria used to experimentally infect feet. The MIC of the organisms re-isolated from infected feet immediately prior to administration of macrolide antibiotic did not differ substantially from those in Table 3.

**Table 3**  
**MIC and MBC of Macrolide Antibiotic**

	<b>Bacteria</b>	<b>MIC (<math>\mu\text{g/ml}</math>)</b>	<b>MBC (<math>\mu\text{m/ml}</math>)</b>
	<i>Porphyromonas levii</i>	<0.03	> 32
5	<i>Prevotella intermedia</i>	4	32
	<i>Fusobacterium necrophorum</i>	0.13	4

The macrolide antibiotic tested in the experimental model was effective in treating five of six animals (83.3%) with experimentally-induced acute footrot. This occurred despite seemingly unfavorable MBC value for both *Porphyromonas levii* and *Prevotella intermedia*. This antibiotic is concentrated within monocytic cells, such as macrophages, at levels much higher than those found in serum and tissue. The ability of these tissue macrophages to kill bacteria may be accentuated in the presence of intracellular antibiotic and this may be a fundamental reason for the success of the antibiotic with bacteria having unfavorable MBC's. Although more data are required to fully establish the usefulness of this antibiotic in bovine footrot, it may be an effective antibiotic for use in this disease based on its performance in the experimental model for this disease.

## EXAMPLE 7

### Immunization of Cattle Study

General Design and Blood Sampling Times: A total of 26 individually identified cattle were used for this study. They were randomly allocated to one of the following groups: 1] immunized or 2] sham-immunized. Prior to immunization each animal had a venous blood specimen collected from the jugular vein by standard venipuncture technique (day 0). Serum was separated from this blood specimen and stored frozen at -85°C. Animals were immunized on day 1. Blood samples for serum were collected three weeks following initial immunization (day 21) when the animals received a second immunization (booster injection of identical preparation). Seven days following the booster injections (day 28) sera were collected again, and the cattle were



inoculated with viable *Porphyromonas levii* and *Fusobacterium necrophorum*, as described in Example 5. Serum samples were collected again on day 35, day 42 and at six months.

Vaccination: Cattle were immunized with: 1] an inactivated whole cell vaccine composition (bacterin) composed of a snap-frozen:thawed preparation of *Prevotella intermedia* and *Porphyromonas levii*, as described above, and alum adjuvant or 2] saline and alum adjuvant. The bacterin contained approximately 0.5 to 0.75 mg protein per ml and each animal received about 1 ml of bacterin containing about 0.75 mg of protein prepared with commercially available alum-based adjuvant by a subcutaneous route. Animals were immunized or sham-immunized on day 1 and day 21. The bacterins were assessed for IgG protease activity by the methods outlined in Example 3 and for  $\beta$ -lactamase activity using nitrocefin discs, as described in Example 4. The *P. levii* bacterin was found to have protease activity but no  $\beta$ -lactamase activity, while the *P. intermedia* bacterin was found to have  $\beta$ -lactamase activity but no protease activity.

Clinical Response: The individual animals were the experimental unit. Clinical scores were assigned daily to animals, using the system described in Example 5, by an individual blinded to experimental group. Cattle that were vaccinated had lower clinical scores than sham vaccinated controls during experimentally induced infection.

This study demonstrated that pre-exposure immunization with *Porphyromonas levii* and *Prevotella intermedia* resulted in the production of serum antibody that were protective in experimental footrot infections. The serum antibody specifically reacting to *Porphyromonas levii* and *Prevotella intermedia* was more abundant in immunized animals than in sham-immunized animals. In particular, levels of serum IgG specific for *P. levii* were significantly higher than the levels in sham vaccinated animals both during and following experimental infection, i.e., between day 28 and 187. The serum antibodies reactive to *Fusobacterium necrophorum* were similar in groups of immunized animals and sham-immunized animals during the entire duration of the experiment (all time points sampled). The antibodies produced by animals immunized with our vaccine conveyed protection from severity of experimental infection compared to sham-immunized animals, as measured by clinical response and scoring. The

severity of experimental infection within individual animals generally correlated with the serum antibody response in those individual animals.

#### EXAMPLE 8

##### 5                   Neutrophil-Mediated Phagocytosis and the Production of                     Immunoglobulin G<sub>2</sub> Protease by *Porphyromonas Levii*

Methods: PMN were purified from whole bovine blood, quantified, assessed for viability, and exposed to one of three putative etiologic agents of AIP (*Porphyromonas levii*, *Prevotella intermedia*, *Fusobacterium necrophorum* ATCC  
10 27852) or latex beads and assessed for phagocytosis using direct microscopy. The effects of bovine serum specifically raised against *P. levii* on antibody-mediated phagocytosis by PMN (opsonization) were examined. *P. levii* was examined for the presence of protease activity capable of cleaving bovine Ig (IgG, IgM, IgG<sub>1</sub>, IgG<sub>2</sub>).

Blood Collection and PMN Purification: Whole bovine blood was collected in  
15 acid citrate dextrose by jugular venipuncture. The erythrocyte fraction was separated by centrifugation and this fraction subjected to repeated cold hypotonic lysis [38]. PMN purified in this fashion were pelleted by centrifugation, resuspended in sterile pyrogen-free PBS, enumerated by haemocytometry, viability determined by staining with 0.1% trypan blue, and assessed for differential leukocyte population on cytopspin  
20 preparations. Preparations were required to be a minimum 95% pure and 95% viable for assays to be performed.

Antibody Production and Evaluation: A calf with low pre-exposure titres to all three bacterial antigen preparations was used. *P. levii* was grown in anaerobic conditions, killed with 10% buffered formalin, washed three times, suspended at 1.0  
25 mg protein per mL, and mixed with alum adjuvant prior to immunization of the calf. The calf received a booster injection 21 days later with the appropriate antigen preparation. Antibody titres were determined by ELISA using killed whole cells of *P. levii* as the antigen preparation.

Phagocytosis Assays: Purified viable PMN ( $1.0 \times 10^6$  cells/mL) were incubated for 15 min ( $37^\circ\text{C}$ ) with  $1.5 \times 10^7$  bacteria/mL following pre-exposure of bacteria to either sterile pyrogen-free PBS, low titre serum, or high titre serum for 30 min. The mixture was centrifuged to pellet PMN, the cells re-suspended in PBS, and the solution cytopinned and stained with Diff-Quik stain for microscopic viewing. Counts of PMN cells phagocytosing bacteria per total cells were determined and photographs were taken.

Protease Preparation, Ig Biotinylation, and Ig Protease Assay: Supernatant of a broth culture of *P. levii* grown in anaerobic conditions (5%  $\text{H}_2$ : 5%  $\text{CO}_2$ : 90%  $\text{N}_2$ ) in supplemented cooked meat broth (haemin, Vitamin K) at  $37^\circ\text{C}$  for four days was collected by centrifugation and stored ( $-80^\circ\text{C}$ ) until used in the protease assay. Culture purity was assured by streak plating onto BBA and standard bacteriological identification.

N-hydroxy-succinimidobiotin was dissolved in dimethyl sulfoxide and was added to each of bovine IgG, IgM, IgG<sub>1</sub> and IgG<sub>2</sub> (1:10 wt/vol), the Igs were incubated for 2h ( $25^\circ\text{C}$ ), and the reaction terminated with 1 mg of glycine. The biotinylated Igs were dialyzed 14h in three changes of double distilled water and final protein concentrations were determined by Bradford assay [39].

The protease assay was a slight modification of the methods of Lee and Shewen [24]. Biotinylated IgG (10  $\mu\text{g}$ ) was added to 250  $\mu\text{L}$  of supernatant and 2.5  $\mu\text{L}$  of penicillin-streptomycin solution (penicillin G 100 U/mL, streptomycin 100  $\mu\text{g}/\text{mL}$ ). The protease assay was carried out at 30, 35 and  $40^\circ\text{C}$  and optimal assay temperature was determined to be  $35^\circ\text{C}$ . Samples (10  $\mu\text{L}$ ) were taken at 0, 0.5, 1, 2, 4, 8, 24, 48, and 72h and added to 10  $\mu\text{L}$  of SDS PAGE sample buffer [30], boiled for 4 min, followed by electrophoretic separation by SDS PAGE. Control assays included *P. levii* supernatant heated to  $100^\circ\text{C}$  prior to exposure to the biotinylated Igs as well as uninoculated cooked meat medium and distilled water.

All samples were resolved in 15% SDS PAGE gels using a 4% stacking gel and standard methodology. Gels were run on ice at 150V for 15 min followed by 200V for 45 min in 10% running buffer. Protein in the gels was transferred to nitrocellulose [31]

at 20V for 14h at 4°C, washed in blocking solution (1h at 37°C), washed with TBS-tween buffer (3 x 15 min 20°C), and incubated with streptavidin-alkaline phosphatase (1:5000) in TBS-tween for 2h at 37°C. Blots were again washed three times for 15 min in TBS-tween and nitroblue tetrazolium (NBT) and 5-bromo- 4 chloro- 3 indolyl phosphate (BCIP) were added as substrate for color development (4 min) in buffer (31.52 g Tris-HCL, 0.18 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 1 l distilled water, pH 8.8). BCIP was initially dissolved in DMSO. Protein bands representing the light and heavy chains of the biotinylated immunoglobulins, or fragments of these immunoglobulin chains, were viewed based on alkaline phosphatase activity on the nitrocellulose. Photographs were obtained immediately.

Results of Phagocytosis Assays: Comparisons of the ability of bovine PMN to phagocytose each of the putative etiologic agents of AIP are shown in Table 4. Values are expressed as mean cells phagocytosing bacteria per 100 PMN  $\pm$  SD (n value is 9 per data point). \* = significantly different than all other experimental groups (P<0.05). Bovine PMN readily phagocytose *P. intermedia* and *F. necrophorum*, but the ability of the cells to phagocytose *P. levii* was significantly reduced compared to these groups and to PBS controls (P< 0.05).

**Table 4. Comparison of Phagocytosis by Bovine PMN *in vitro* of Latex Beads, *P. levii*, *P. intermedia* and *F. necrophorum***

Experimental Group	Phagocytosis
Latex beads	17.9 $\pm$ 3.8
<i>P. levii</i>	5.3 $\pm$ 1.6*
<i>P. intermedia</i> (8-2)	60.7 $\pm$ 6.1
<i>F. necrophorum</i>	64.1 $\pm$ 4.4

The phagocytic abilities of PMN for *P. levii* when the bacteria were pre-incubated with PBS, low titre anti-*P. levii* serum, high titre anti-*P. levii* serum, or complement-destroyed high titre anti-*P. levii* serum were examined. High levels of phagocytosis were

evident in these same cell preparations if the *P. levii* were pre-incubated with high titre anti-*P. levii* bovine serum. This restoration of phagocytic function was not altered substantially by heating the serum at 56°C for 30 min (Table 5). \* = significantly greater than both PBS control and low titre serum ( $P < 0.05$ ) but not different from complement destroyed serum.

**Table 5. Examination of Phagocytosis Abilities of PMN for *P. levii* when Incubated with Anti-*P. levii* Serum**

	Experimental Group	Phagocytosis
10	<i>P. levii</i> and PBS	5.3 $\pm$ 1.6
	<i>P. levii</i> and low Ig	30.0 $\pm$ 5.5
	<i>P. levii</i> and high Ig	97.0 $\pm$ 1.1*
	<i>P. levii</i> and inact. complement factors	89.9 $\pm$ 3.8*

15 Ig Protease Assays: Nitrocellulose transfer of biotinylated IgG exposed to culture supernatant of *P. levii* is shown in Fig. 9A. From left to right are increasing times (hours) of exposure. Lane A = 0, Lane B = 0.5, Lane C = 1, Lane D = 2, Lane E = 4, Lane F = 8, Lane G = 24, Lane H = 48, and Lane I = 72h time samples. Results show the presence and increasing intensity of the degradation bands (major bands are highlighted  
20 with arrows) smaller than the heavy and light chains (arrowheads) as incubation time increases. Also, near complete degradation of the heavy chain by 24h was noted.

In contrast, nitrocellulose transfer of biotinylated IgM exposed to culture supernatant of *P. levii* is shown in Fig. 9B. From left to right are increasing times (hours) of exposure. Lane A = 0, Lane B = 0.5, Lane C = 1, Lane D = 4, Lane E = 8, Lane F =  
25 24, Lane G = 48, and Lane H = 72h time samples. There is absence of degradation bands. Only the light and heavy chains (arrowheads) of IgM are evident.

When IgG<sub>1</sub> was assayed, degradation bands were not evident (Fig. 10A) and again only bands representing the heavy and light Ig chains could be seen. However, when assays using IgG<sub>2</sub> were conducted similar results to those employing crude IgG were seen

with degradation bands appearing within 30 min, as illustrated in Fig. 10B, but heavy chain was not as extensively degraded. Controls using water alone, heated supernatant, supernatant extracts of *P. intermedia* or *F. necrophorum*, or uninoculated media (each of which was exposed to the biotinylated IgG) showed no evidence of degradation bands  
5 (data not shown).

Modification of the above-described modes of carrying out the various embodiments of this invention will be apparent to those skilled in the art following the teachings of this invention as set forth herein. The examples described above are not limiting, but are merely exemplary of this invention, the scope of which is defined by  
10 the claims which follow.

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Claims:

1. A composition for preventing or treating footrot comprising:
  - a) at least one immunogen selected from the group consisting of *Prevotella*, *Porphyromonas*, a subunit of *Prevotella* or *Porphyromonas* and a toxin of *Prevotella* or *Porphyromonas*; and
  - b) a pharmaceutically acceptable carrier.
2. The composition of Claim 1 wherein the immunogen is selected from the group consisting of *Prevotella*, *Porphyromonas*, a toxin of *Prevotella*, a toxin of *Porphyromonas*, *Porphyromonas levii*, *Prevotella intermedia*, a protease, an immunoglobulin protease, an IgG<sub>2</sub> protease of *Porphyromonas levii* and a  $\beta$ -lactamase of *Prevotella intermedia*.
3. The composition of Claim 1 wherein the immunogen is an immunoglobulin protease and the immunoglobulin is selected from the group consisting of IgG and IgM.
4. A method of preparing the composition of Claim 1 wherein the immunogen is *Prevotella*, or *Porphyromonas* comprising:
  - a) culturing *Prevotella* or *Porphyromonas* anaerobically;
  - b) recovering *Prevotella* or *Porphyromonas* cells from the culture;and
  - c) adding a pharmaceutically acceptable carrier.
5. The method of Claim 4, further comprising the step of inactivating the cells.
6. A method of preparing the composition of Claim 1 wherein the immunogen is a toxin of *Prevotella* or *Porphyromonas* comprising:
  - a) cultivating *Prevotella* or *Porphyromonas* anaerobically;

- b) recovering the supernatant from the culture;
- c) isolating the toxin from the supernatant; and
- d) adding a pharmaceutically acceptable carrier.

5           7.     The method of Claim 6, further comprising the step of inactivating the  
toxin.

8.     A method of preventing or treating footrot in an animal suffering from or  
susceptible to footrot comprising administering to said animal an effective amount of  
10    the composition of Claim 1.

9.     The method of Claim 8 wherein said composition is administered  
parenterally or orally.

15       10.    The method of Claim 8 wherein said administration is parenteral  
administration selected from the group consisting of subcutaneous, intramuscular,  
intraperitoneal, intranasal, local (intrapodal), and intravenous administration.

11.    The method of Claim 8 wherein said administration is performed two or  
20    more times.

12.    The method of Claim 11 wherein said administration comprises annual  
administration.

25       13.    The method of Claim 8 wherein said effective amount is sufficient to  
prevent symptoms of footrot.

14.    The method of Claim 8 wherein said effective amount is sufficient to  
inhibit the function of at least one agent selected from the group consisting of proteases

of bacteria associated with footrot, an IgG<sub>2</sub> protease of *Porphyromonas levii* and a  $\beta$ -lactamase of *Prevotella intermedia*.

15. A toxin of *Prevotella* or *Porphyromonas* isolated from a strain of  
5 *Prevotella* or *Porphyromonas*.

16. The toxin of Claim 15 wherein immunization with said toxin prevents symptoms of footrot when an effective amount of said toxin is administered to an animal.  
10

17. The toxin of Claim 15 which is selected from the group consisting of a protease, an IgG<sub>2</sub> protease of *Porphyromonas levii*, a lactamase and a  $\beta$ -lactamase of *Prevotella intermedia*.

18. A method of producing a toxin of *Prevotella* or *Porphyromonas* comprising:  
a) preparing a culture of *Prevotella* or *Porphyromonas* by growing the bacteria anaerobically; and  
b) isolating said toxin from said culture.  
20

19. An antibody to at least one selected from the group consisting of *Prevotella*, *Porphyromonas*, a subunit of *Prevotella* or *Porphyromonas* and a toxin of *Prevotella* or *Porphyromonas*.

20. The antibody of Claim 19 which neutralizes an isolated toxin of *Prevotella* or *Porphyromonas*.  
25

21. The antibody of Claim 19 which, when administered to an animal showing symptoms of footrot, alleviates said symptoms.  
30

22. A method of providing passive immunity in an animal susceptible to or suffering from footrot comprising administering to said animal an effective amount of the antibody of Claim 19.

5           23. An experimental model useful to evaluate effectiveness of vaccines or treatments for bovine footrot comprising a bovine which has been inoculated in the subcutaneous interdigital area with *Prevotella* or *Porphyromonas*.

          24. The model of Claim 23 wherein said *Prevotella* or *Porphyromonas* is  
10 administered in conjunction with *Fusobacterium necrophorum*.

          25. A composition for preventing or treating footrot comprising:  
          a) at least one agent which neutralizes at least one selected from the  
group consisting of *Prevotella*, *Porphyromonas*, a subunit of *Prevotella* or  
15 *Porphyromonas* and a toxin of *Prevotella* or *Porphyromonas*; and  
          b) a pharmaceutically acceptable carrier.

          26. The composition of Claim 25 wherein said agent is an antibody to at  
least one selected from the group consisting of *Prevotella*, a subunit of *Prevotella* and a  
20 toxin of *Prevotella*.

          27. The composition of Claim 25 wherein said agent neutralizes a bacterial  
protease.

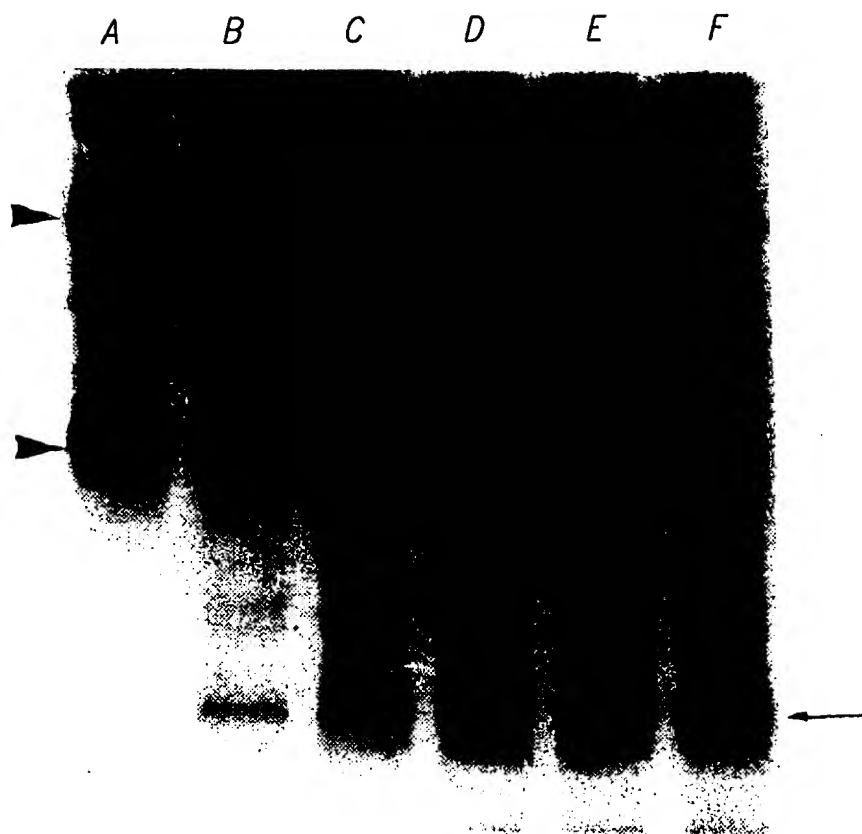
25           28. A method for preventing or treating footrot in an animal suffering from or susceptible to footrot comprising administering to said animal an effective amount of a composition which enhances neutrophil-mediated resolution of infection by bacteria associated with footrot.

29. The method of Claim 28 wherein said resolution of infection is at least one mechanism selected from the group consisting of phagocytosis and oxidative metabolism.

5           30. The method of Claim 28 wherein said composition is the composition of Claim 25.

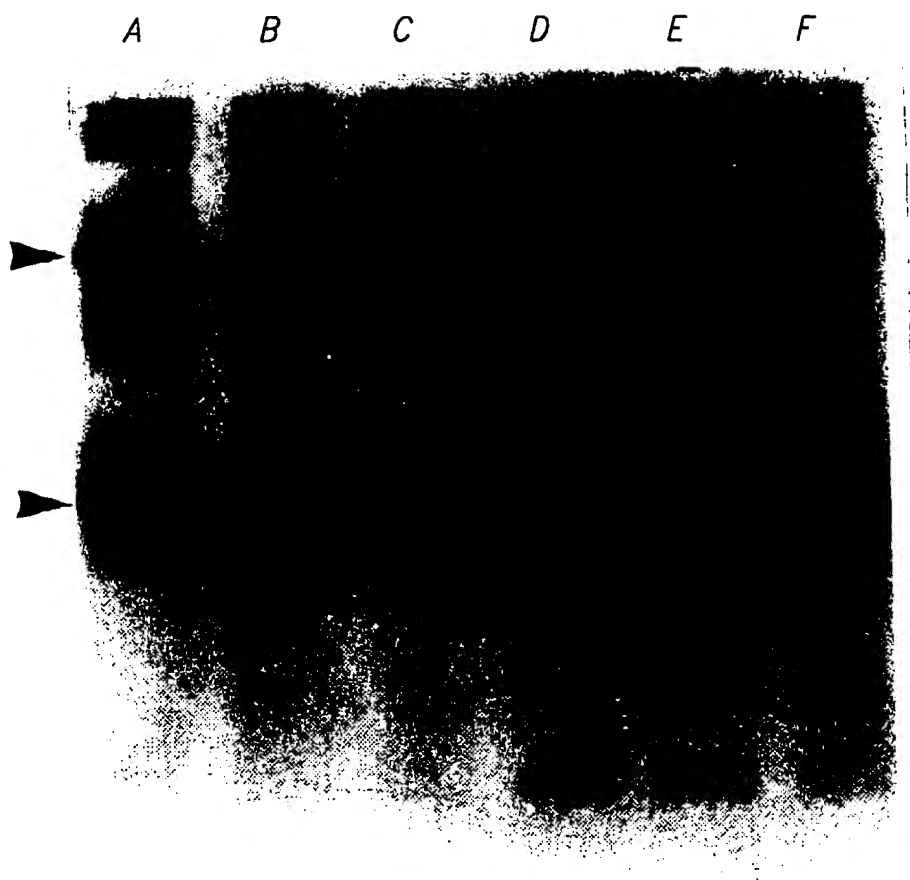
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Figure 1A



2/11

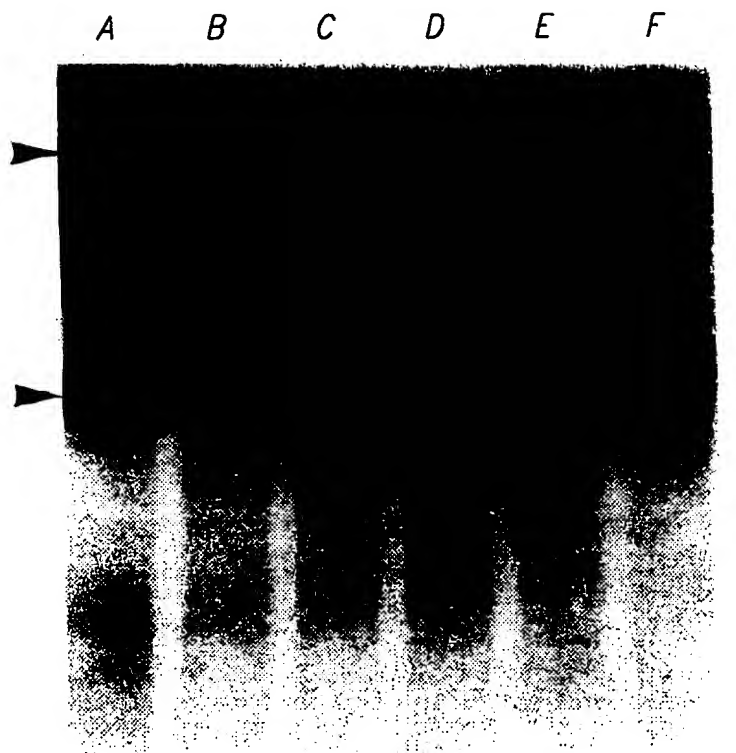
Figure 1B





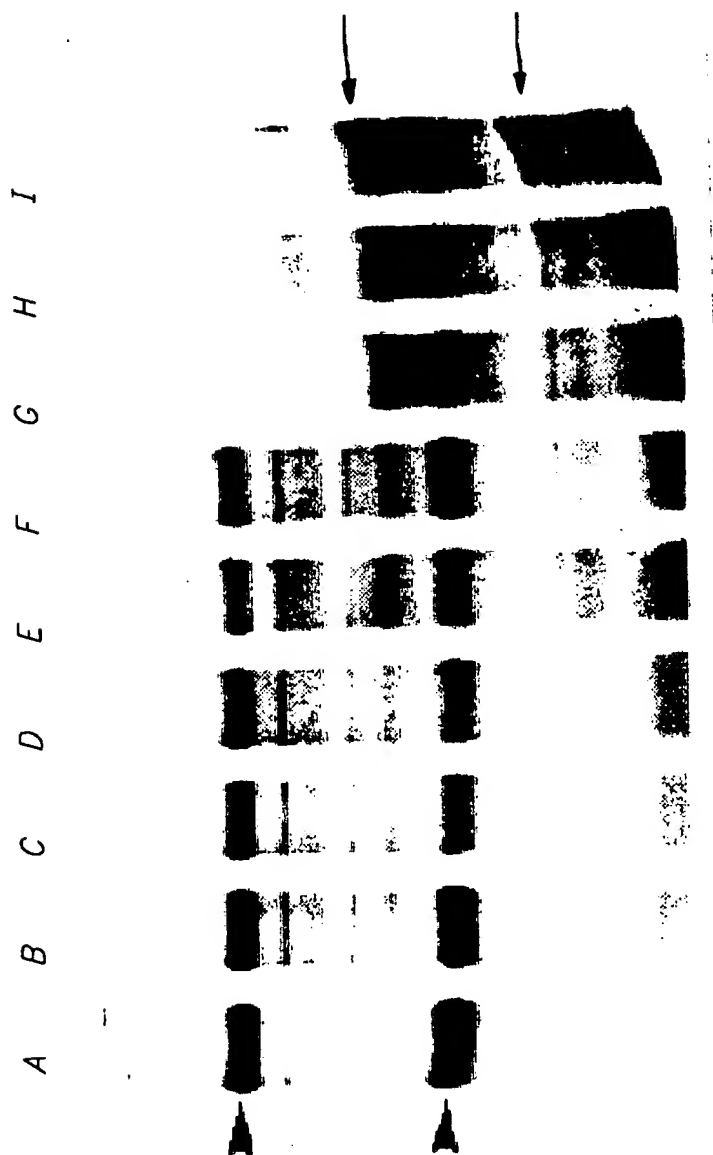
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Figure 1C



4/11

Figure 1D



5/11



Figure 2

6/11

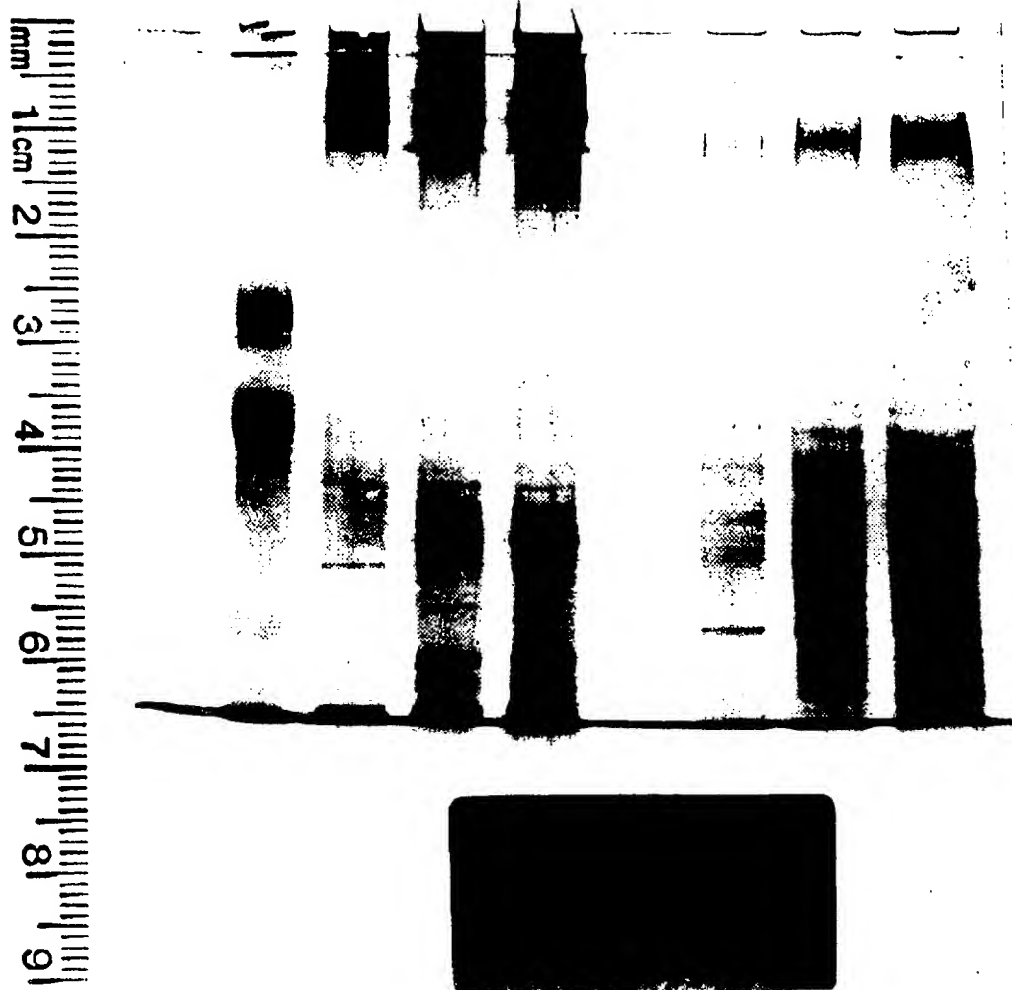


Figure 3

7/11

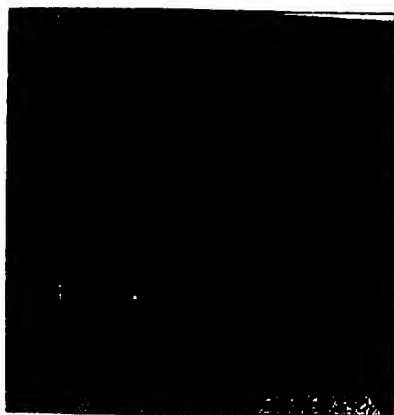


Figure 4A



Figure 4B



Figure 4C

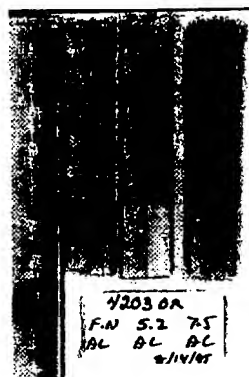


Figure 5A



Figure 5B

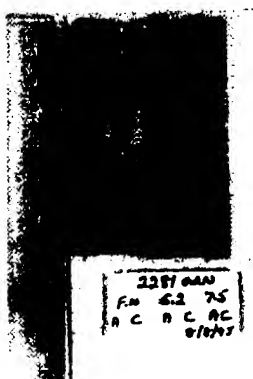


Figure 5C

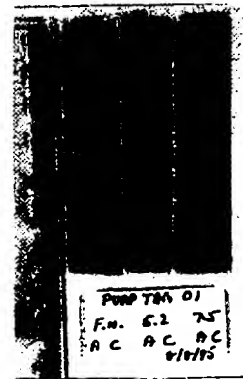


Figure 5D

8/11

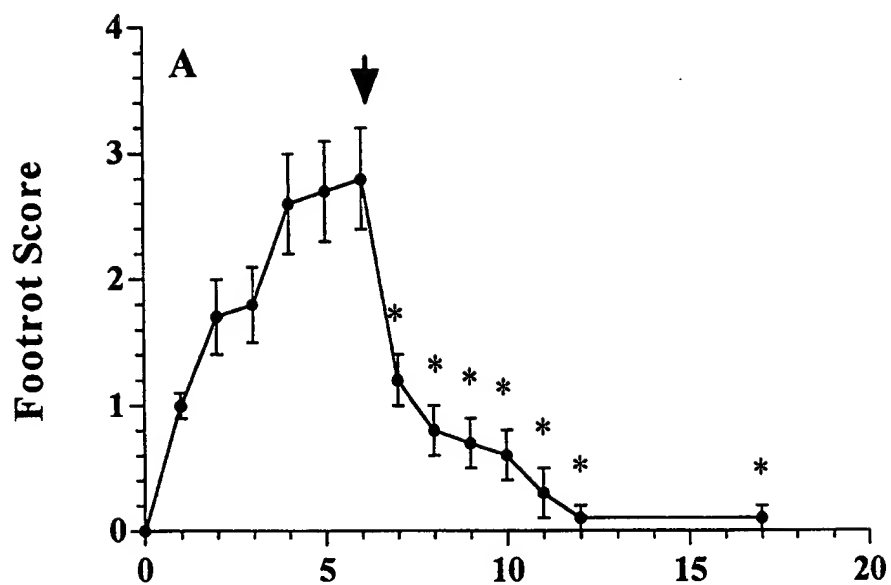


Figure 6A

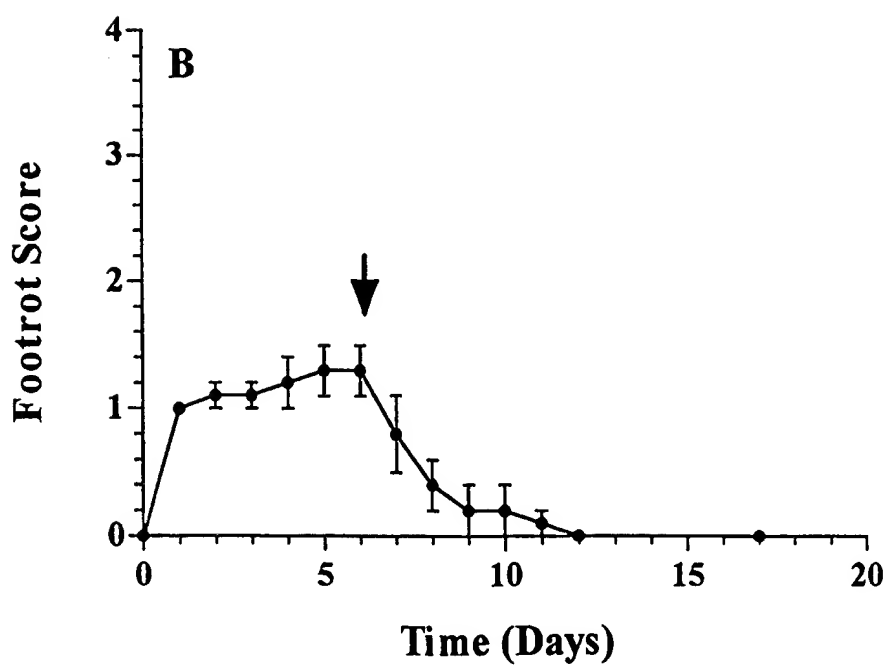


Figure 6B

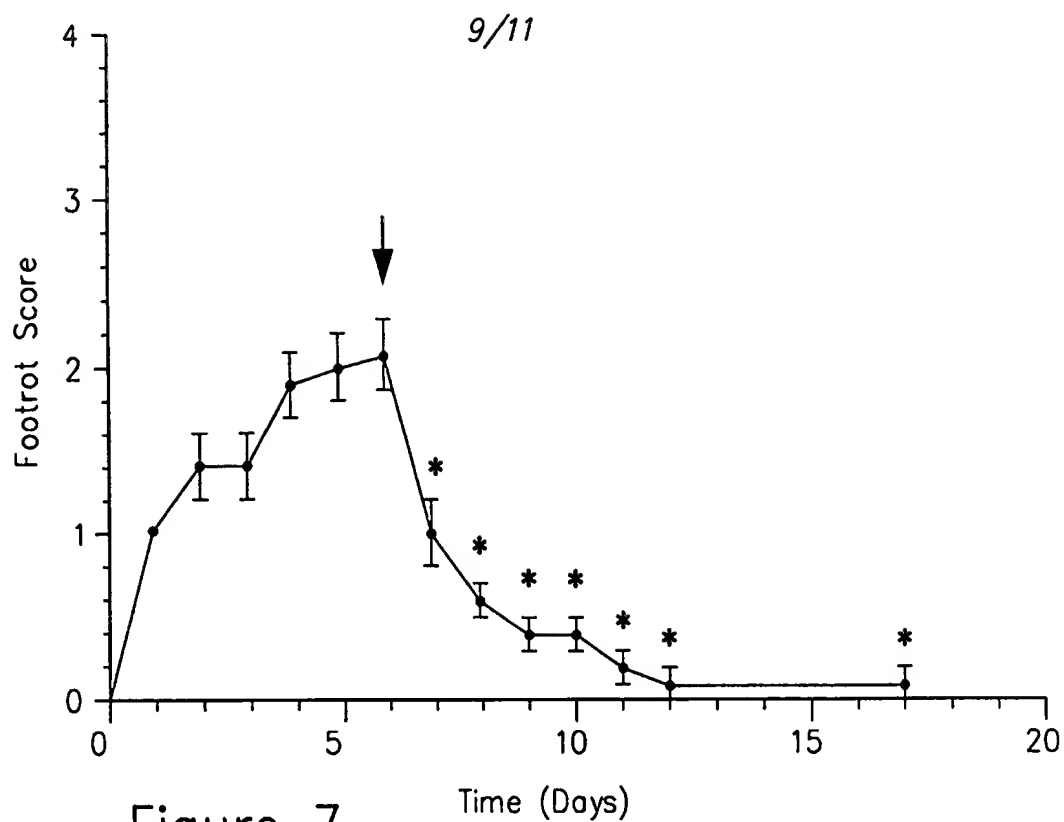


Figure 7

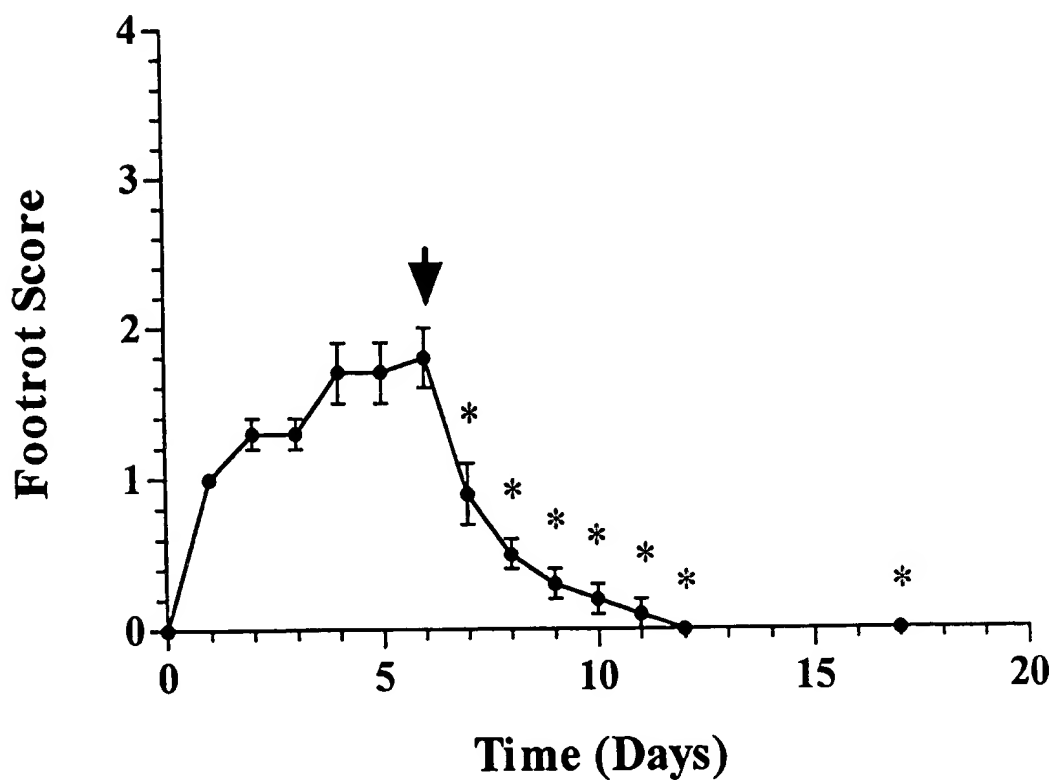


Figure 8

10/11



Figure 9A

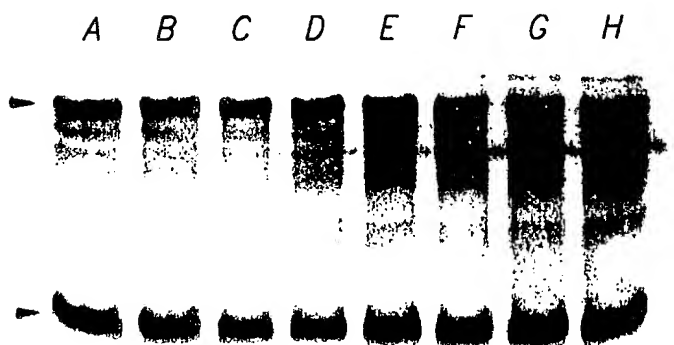


Figure 9B



11/11



Figure 10A

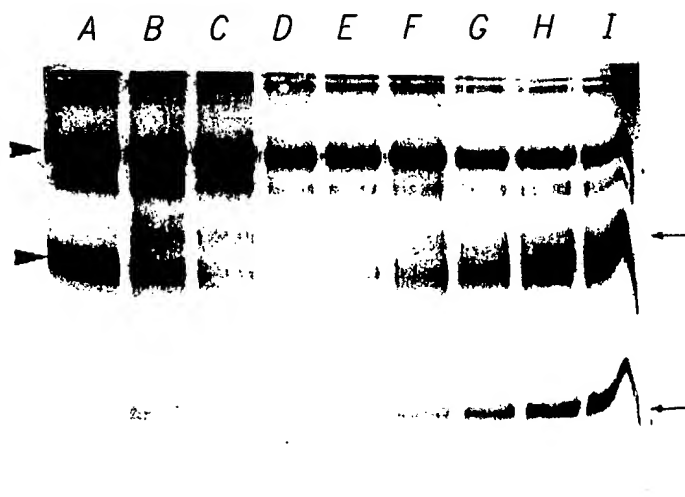


Figure 10B

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 98/00823

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/02 A61K39/40 C07K16/12 C12N1/20 C12N9/52

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 288 617 A (MATTICK J.S. ET AL.) 22 February 1994 see the whole document ---	1-27
A	WO 92 03553 A (DARATECH PTY) 5 March 1992 see the whole document ---	1-27
A	DE 43 24 859 A (LION CORP.) 27 January 1994 see the whole document ---	1-27
A	BERG J N ET AL: "Fusobacterium necrophorum and Bacteroides melaninogenicus as etiologic agents of foot rot in cattle." AMERICAN JOURNAL OF VETERINARY RESEARCH, (1975 AUG) 36 (08) 1115-22, XP002088079 see the whole document ---	1-27
-/--		



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search

16 December 1998

Date of mailing of the international search report

13/01/1999

Name and mailing address of the ISA

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Moreau, J

# INTERNATIONAL SEARCH REPORT

In tional Application No

PCT/CA 98/00823

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MORCK D W ET AL: "Experimental evaluation of a commercial footrot vaccine against native Canadian strains of Dichelobacter nodosus." CANADIAN JOURNAL OF VETERINARY RESEARCH, (1994 APR) 58 (2) 122-6, XP002088080 see the whole document ----	1-27
A	SPARROW, D. G. ET AL: "Expression of tumor necrosis factor.alpha. and granulocyte- macrophage colony-stimulating factor by bovine endothelial cells in vitro" CLIN. INFECT. DIS. (1997), 25(SUPPL. 2, PROCEEDINGS OF THE 1996 MEETING OF THE ANAEROBE SOCIETY OF THE AMERICAS), S178-S179 , XP002088081 see the whole document -----	28-30

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 98/00823

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 8-14, 22 and 28-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 98/00823

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5288617 A	22-02-1994	AU 590230 B AU 5015485 A WO 8602557 A EP 0202260 A JP 62500933 T	02-11-1989 15-05-1986 09-05-1986 26-11-1986 16-04-1987
WO 9203553 A	05-03-1992	NONE	
DE 4324859 A	27-01-1994	JP 6040869 A JP 6040870 A JP 6040871 A US 5759544 A	15-02-1994 15-02-1994 15-02-1994 02-06-1998

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